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Abit Babu

MOLECULAR BIOLOGICAL METHODS FOR NUCLEIC ACID DETECTION

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Abstract

Nucleic Acid amplification and detection by the molecular biological methods is a technology provide guidance on the diagnosis of genetic material. Every genetic material consists of DNA/RNA but each of them has a variation in genome sequences. An excellent laboratory with special equipment and technical expertise are required for the nucleic acid amplification since the contamination rate is high. The main task of this thesis was to detect eDNA from the fish name as PIKE in an environmental sample. The subtasks include the DNA extraction by booms technology and amplification of the eDNA by three molecular detection method, which is Polymerase Chain Reaction (PCR), Loop-Mediated Isothermal Amplification (LAMP) and Nucleic acid sequence-based amplification (NASBA). This master project also focuses on designing and testing of pike related biomarkers using PCR, LAMP, and NASBA from selected genes. The selected genes included in this study were mitochondria, Histone and GAPDH. A verification study was also conducted comparing the three methods used on the same samples. A fish species named Esox Lucius (also known as Northern Pike) was selected as the experimental subject. Esox Lucius is a species of carnivorous fish belonging to the genus Esox (the pikes). It lives in both marine and freshwater of the Northern Hemisphere (i.e., Holarctic in distribution. Northern pike can attain lengths up to 4.5 feet (137 cm) and weigh up to 62.5 lbs. (28.4 kg)[1]. This research was conducted in association with a future generation micro and nano tech device which is under invention by USN.

Acknowledgement

The work here is a final thesis for my master's degree in micro and nano systems technology from the University of South-Eastern Norway. With the help of this project, I have been able to gain an in-depth understanding of extraction, amplification and detection of DNA/RNA.

In the first place, I'd like to thank Frank Karlsen, my supervisor. Throughout the entire project, he motivated me and gave me good direction whenever I needed it. He also provided many of his research papers which was an additional support my research work. His Support through the entire project helped me to finish the project successfully. I would also like to thank prof. Lars Eric Roseng, lab supervisor Birgitte Kasin Hønsvall and PhD student Nivedhitha Narayanan for guiding and assisting me at numerous points during my work in the lab, they also provided me with equipment to use. Moreover, I would like to thank the University of South-Eastern Norway for providing me with such a valuable master's program, where I was able to work in advanced laboratories, under the guidance of dedicated professors. As coordinators of my course, I also wish to thank Kristin Imenes and Merete Elisabeth Hovet for guiding me throughout my master's program.

Finally, I am very grateful to my parents and friends for their unconditional support and continued encouragement throughout my years of study and during the research and writing process of this thesis.

Table of contents

List of Tables

List of Figures

Figure 44: Amplification plot for NASBA with GAPDH primers on oligos

List of Acronyms

DNA – Deoxyribonucleic acid

eDNA- environmental Deoxyribonucleic acid

RNA- Ribonucleic acid

mRNA- Messenger Ribonucleic acid

eRNA- environmental Ribonucleic acid

PCR – Polymerase Chain Reaction

LAMP – loop mediated isothermal amplification

NASBA – Nucleic acid sequence-based amplification

POCNAD – Point of care Nucleic Acid Detection

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase

NCBI – National Center for Biotechnology Information

cDNA- complementary DNA

Mito- Mitochondria

1.0 Introduction

Motivation

Environmental DNA refers to the presence of genetic material in a sampled milieu in the absence of a proximate biological source (Thomsen and willerslev,2015)[2]. The analysis of eDNA has quickly become a powerful tool for improving species detection and uncovering patterns of biological diversity. eDNA/eRNA amplification and detection has become an important factor in the present world. It will help in identifying the disease and extracting the data from the sample. For the amplification and detection, primers serve as an initiator for DNA synthesis and probes are used to detect nucleotide sequence which is complementary to the sequence. Primers and probes are the biomarkers used in this project. Biomarkers were introduced in 1950s.They were defined as "measurements at the molecular, biochemical, or cellular level in either wild populations from contaminated habitants and the magnitude of the organism's response"(Mc Carthy and Shugart,1990).It was also defined as "biochemical and/or physical changes in organism exposed to contaminants and thus represent initial responses to environment perturbation and contamination"(De Lafontaine,2000).Biomarker application includes disease detection, diagnosis, prognosis, prediction of response ,intervention and disease monitoring[3].

There are lot of tests has been developed for the diagnosis of the pathogens to detect and fight against the causative agents of diseases. But these tests are unsatisfactory, and they are not sensitive, specific, and unable to deliver a quick result. Nucleic acid test has showed promising result to overcome the limitations[4]. PCR, LAMP and NASBA are some of the most popular nucleic acid-based diagnostics used in this dissertation. PCR technique was patented by Kerry Mullis and assigned to Cetus corporation in 1983. In 1989 an agreement was signed between the cetus corporation and Roche for the development diagnostics of the polymerase chain reaction[5]. Because of that nucleic acid amplification become important (Heilek, 2016). Within this concept, automated laboratory platforms have been designed to facilitate the workflow and to ensure accurate and precise examination of samples. Initially, PCR was used for the automation of these platforms (Straub et al., 2005). This thesis focuses on the amplification and detection of the eDNA by these three molecular diagnostic methods for the selected genes that we designed and produced in USN laboratory for the nucleic acid test platform.

Contribution of the thesis

In terms of diagnostic methods, nucleic acid tests are an important and accurate method that provides clear results. It is also essential that such methods are integrated into the platforms and automated. In the future, molecular tests may be performed more efficiently and with less complexity, thereby reducing the volume of personnel and training required. In this thesis, three molecular tests are explained in detail with real time monitoring of the amplification curves. PCR is the oldest and most well-known method for detecting eDNA, while LAMP and NASBA are two new methods. Many good PCR primers and probes for the detection of various biomarkers are known, while good LAMP and NASBA primers and probes are little known. With the lamp, DNA extraction protocols can be fast, minimal, and require relatively inexpensive equipment and Bst polymerase, which is highly resistant to reaction inhibitors (Kubota et al., 2011). to verify brand new LAMP and NASBA primers and probes.

Thesis outline

Introduction gives a brief knowledge about the major terms in this master's thesis. Definition of DNA and RNA are explained to provide a basic understanding of these terms which is essential for this project. Three gene were selected from mitochondria, histone and GAPDH for designing the primers are probes. A short description of the method "Booms Extraction" which is used as a method for the extraction of DNA/RNA has been explained. Primers and probes are collectively called biomarkers which is an essential part of this project were also defined. The most important part of the project are the methods used for amplification and detection. A brief note about PCR, LAMP and NASBA has been explained in the theory part. Finally, a short note on the Software used for the designing of the biomarkers were outlined.

2.0 THEORY

2.1 DNA AND RNA

DNA and RNA are the two main types of nucleic acids. An organism's genetic instructions are carried by a molecule called DNA. It is made up of two strands that wind around one another to form a double helix. The backbone of each strand is comprised of sugar (deoxyribose) and phosphate groups alternately. Sugars are attached to four kinds of bases - adenine (A), cytosine (C), guanine (G), and thymine (T). The two strands are held together by bonds between the bases: adenine bonds with thymine, and cytosine bonds with guanine. The sequence of bases along the backbone serves as a guide for assembling protein and RNA molecules [6].

RNA is a short single stranded ribonucleic acid like DNA. Transcription of RNA from DNA is accomplished by enzymes called RNA polymerases, which are further processed by other enzymes. It is needed for protein synthesis. An RNA called messenger RNA carries information from DNA to structures called ribosomes. RNA and proteins make up the ribosomes, which is a machine that can read and translate messenger RNAs into proteins [7]. There are four different bases in ribonucleic acid (RNA), including A, U, C, and G, in addition to a phosphate group and a sugar molecule (ribose). Thus, RNA molecules have the same building blocks as DNA molecules. The RNA molecule differs from DNA in that it has the base U instead of T, and the sugar ribose instead of deoxyribose [8].

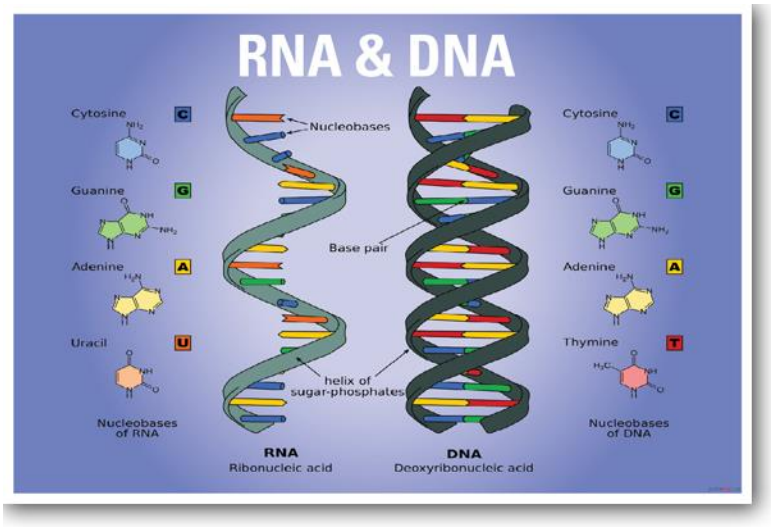

Figure 1: structure of DNA and RNA and their differences[1][9].

2.1.1Mitochondria

Cells contain tiny organelles called mitochondria that inhabit the cytoplasm, the fluid-filled space between the cell nucleus and the outer membrane. Mitochondria contain their own genome. Each cell contains thousands of mitochondria, and each one has its own small circle of DNA. This is a reminder of their distant bacterial ancestry. Mitochondrial DNA encodes genetic information in the mitochondria [10]. It is the smallest genome known to science. Genetically, the genome has no non-coding regions, and genes lack many of the features normally associated with eukaryotic genes. The figure 2 shows the different genes and proteins present in the whole mitochondrial DNA.

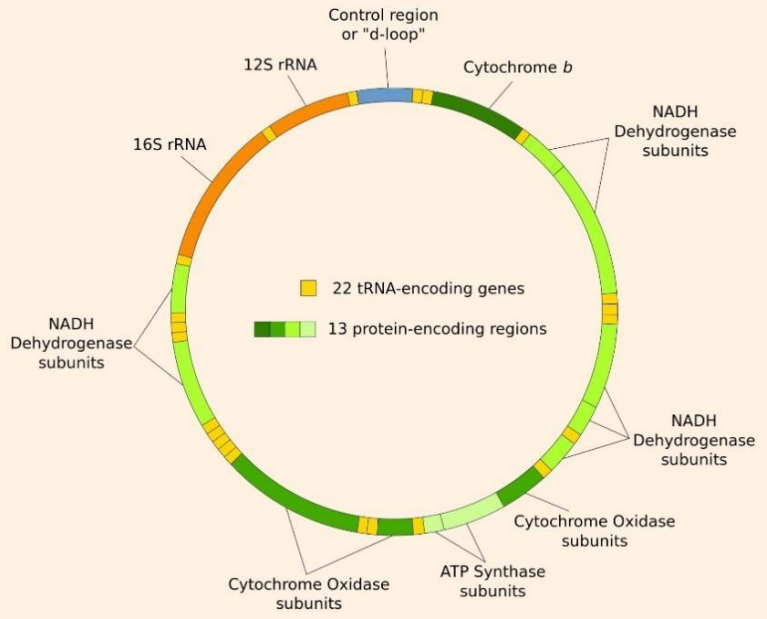

Figure 2: Map of mitochondrial genome [11]

Since it is in more quantity and availability in tissue, it is considered as the laboratory experimental genome. Cytochrome b oxidase gene of mitochondrial DNA is used for the creation of primers

[1] *three differences between DNA and RNA are that DNA uses the base thymine while RNA uses uracil, DNA uses the sugar deoxyribose while RNA uses ribose, and usually DNA is double-stranded, and RNA is single-stranded.*

and probes for the PCR and LAMP test. And cytochrome Oxidase subunits genes were used for NASBA.

2.1.2 Histone

DNA in the nucleus is condensed into chromatin by the proteins known as histones; they are alkaline (basic pH) proteins, and they can associate with DNA because of their positive charges. These are found inside the nucleus of eukaryotic cells. Nucleosomes are made up of DNA and histones; nucleosomes break down into chromatin; two chromatins make up a chromosome. Five types of histones have been identified: H1 (or H5), H2A, H2B, H3 and H4, the core histones are H2A, H2B, H3, and H4, and the linker histones are H1 and H5. A histone's main function is to compact DNA strands and to regulate chromatin [12].

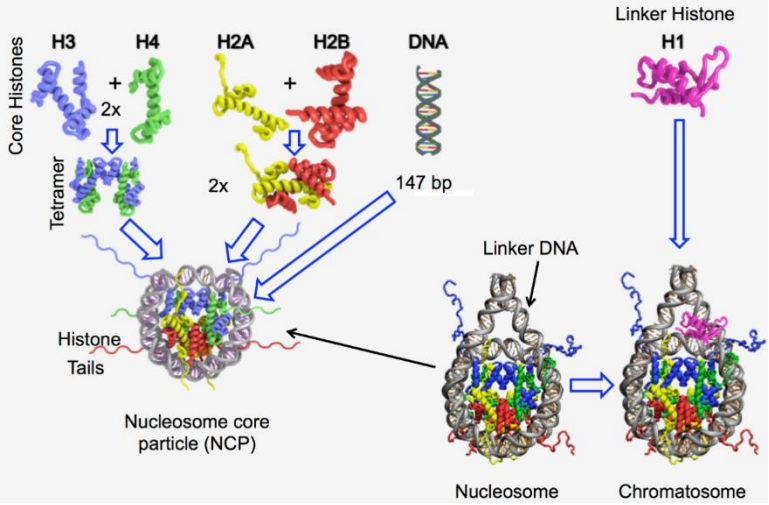

Figure3: Assembly of the histone variants [12].

2.1.3 Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is one of the most widely used genes in gene expression studies. It is an enzyme of about 37 kDa that participates in the sixth step of glycolysis, allowing glucose to be broken down for energy and carbon molecules. Since the GAPDH gene is expressed at high levels in most tissues and cells, it is considered a housekeeping gene. Therefore, it is commonly used by biological researchers as a loading control in western blots and quantitative polymerase chain reactions. However, researchers have reported different

regulatory mechanisms for GAPDH for different conditions. It is also an enzyme highly conserved within the glycolytic pathway. GAPDH plays other roles in the cell that result in variable amounts of expression across different tissues [14]. Studies have shown a variety of functions and activities of this protein ranging from DNA and RNA binding plays key roles in neurodegenerative diseases such as Alzheimer's [15].

2.2 Booms Extraction

The booms method is a solid phase extraction method for obtaining nucleic acid from a biological sample. The silica beads are used to absorb the nucleic acids [16]. Boom method involves four steps for the isolation of nucleic acid. Lysing and/or homogenizing the sample, addition of silica beads to bind the NA to the silica beads, washing the silica beads and finally, elution. This method depends on the nucleic acid-binding properties of silica particles or diatoms present in the presence of the chaotropic agent, which are related to the chaotropic effect. The chaotropic effect is where a chaotropic anion in an aqueous solution disturbs the structure of water and weakens the hydrophobic interaction [17, p.495].

2.3 Primers and Probes

A primer is a short single-stranded DNA sequence used as a catalyst in amplification techniques for a specific gene. A pair of primers is used to hybridize with the sample DNA and define the region of the DNA that will be amplified. Primers are also referred to as oligonucleotides. It has a length ranging from 18 to 24 base pairs. It can also be used for several other experiments. For example, you might use a primer for sequencing a reaction in which you would like to concentrate on a very specific region and then analyze the extension of the DNA molecule [18].

A probe is a strand of DNA or RNA with a label attached. Labeling allows us to see where DNA binds, either within a cell or within chromosomes or even in pure DNA. Various molecules are used to label probes. For chemically attaching it to a probe, radioactive or fluorescent materials can be used. This probe can be then used to see where particular mRNAs are expressed in a cell or a tissue. Probes can also be used to screen the genome for extra copies, which occur frequently in cancers, or missing copies, which occur in hereditary syndromes and cancers [19].

2.4 Methods for Amplification and Detection of DNA/RNA

Detecting DNA variations and their variations is crucial for a wide range of investigations, such as veterinary and clinical diagnostics, industrial and environmental testing, and agricultural studies. To diagnose and predict disease conditions (e.g., cancer), infectious organisms (e.g., HIV), and genetic markers, it is necessary to detect them effectively. The analysis of DNA from original specimens, however, is a complicated process involving multiple chemical compounds as well as several redox processes. Yet, DNA analyte concentrations in test samples are not high enough to enable direct detection. Hence, DNA amplification is required to increase the concentration of the target sequence. There are many methods for the amplification and detection of DNA/RNA. Three different methods explained in this thesis.

2.4.1 PCR (polymerase chain Reaction)

Polymerase chain reaction (PCR) is a fast and inexpensive technique used to amplify small segments of DNA. It enables researchers to amplify small sample of DNA to a large enough amount to study in detail, making million or billion copies of it. With PCR, a segment of DNA is amplified by first heating the sample so that it denatures or separates into two pieces of single-stranded DNA. Following that, an enzyme called 'Taq polymerase' synthesizes new strands of DNA using the original template strands. The result is the replication of the original DNA, with each new molecule containing one old and one new strand. By doing so, each strand can be used to create two copies, and so on. More than one billion identical copies of the original DNA segment are created by denaturing and synthesizing new DNA 30 to 40 times [20].

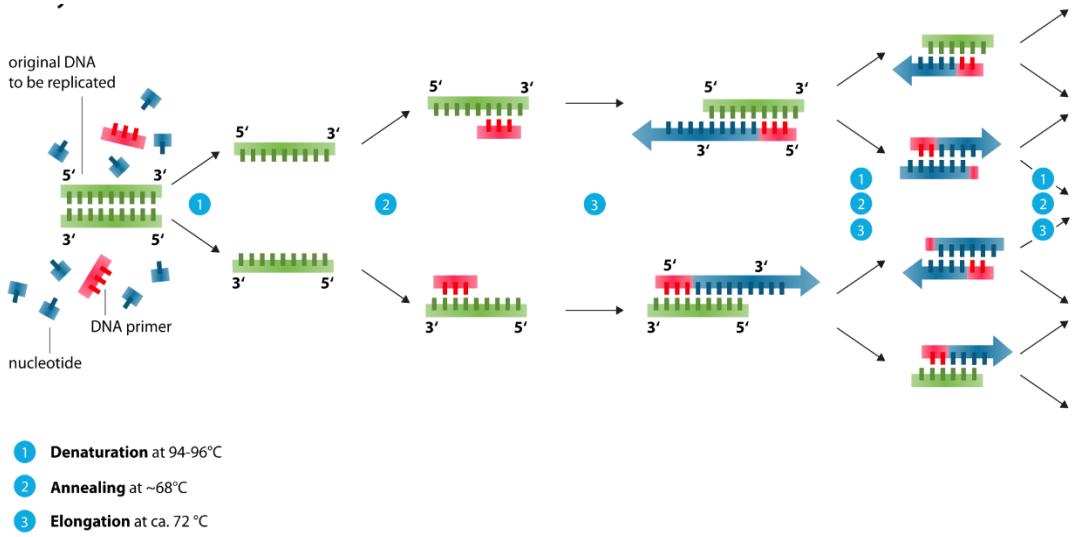

Figure 4: Steps in PCR reaction cycle [21].

The diagram shows the different steps how a PCR works in the whole process. Three main steps involved in the procedure are denaturation, annealing and elongation.

The initial step is the pre-heating of the PCR which is required for the DNA polymerase. The next step is the denaturation of the sample which done at 94–98 °C for 20-30 seconds where the hydrogen bonds between complementary bases are broken, causing denaturation of the double-stranded DNA template, give rise to a single-stranded DNA molecule. In next step the sample is treated at annealing temperature, that is the temperature is lowered to 50–65 °C for 20-40 seconds. It allows annealing of the primers to each of the single-stranded DNA templates. At this step, the polymerase attaches to the primer-template and begins to form DNA.

Next is extension, in this stage the temperature is set according to the DNA polymerase used. Most used temperature is 72 °C with this enzyme. In this step, the DNA polymerase synthesizes a new complementary strand of DNA. Each successive cycle results in exponential amplification of the specific DNA target region due to the addition of the previous template strands plus all newly generated strands. The three-process denaturation, annealing and extension forms a single cycle. Finally, cool down process occurs at 4-15°C for indefinite time [22].

PCR has numerous applications, not only in basic research but also in medical diagnostics, forensics, and agriculture. Hence, PCR is an effective method in detecting DNA and RNA in a

sample but one of the main drawbacks of this process is the potential presences of contaminations. Therefore, PCR performing laborat

ories should be certified to use this technique.

2.4.2 LAMP (loop mediated isothermal amplification)

LAMP (Loop-mediated Isothermal Amplification) is a novel approach to nucleotide amplification developed by Notomi et al. By using the assay, the target gene can be amplified isothermally without the use of expensive thermal cyclers [23]. LAMP uses and with the presence of specific primers and target DNA template at 60-65 °C for 45-60 minutes. It requires 4-6 primers specific to identified regions of target DNA or RNA sequence. Stages of LAMP method (Mori & Notomi, 2020; Nzelu et al., 2019) is shown in figure 5. Amplification initiates from strand invasion by one of the inner primers (FIP/BIP) and a strand displacing DNA polymerase extends the primer and separate the target DNA duplex. The first product is then displaced by synthesis initiating from an outer primer(F3/B3) which anneals to upstream target region. As it is displaced the end of the product forms a self-hybridizing loop structure due to inclusion of a reverse complementary sequence in the inner primer sequence. This annealing and displacement cycle repeats on the opposite end of the target sequence and the resulting product is a short dumbbell structure that forms a seed for exponential lamp amplification. This dumbbell structure contains multiple sites for initiation of synthesis from the prime ends of the open loops and annealing sites for both the inner and loop primers. As amplification proceeds from these multiple sites the product grows and form concatemers each with more sites for initiation. The result is a rapid accumulation of double stranded DNA and amplification byproduct that can be detected by LAMP method.

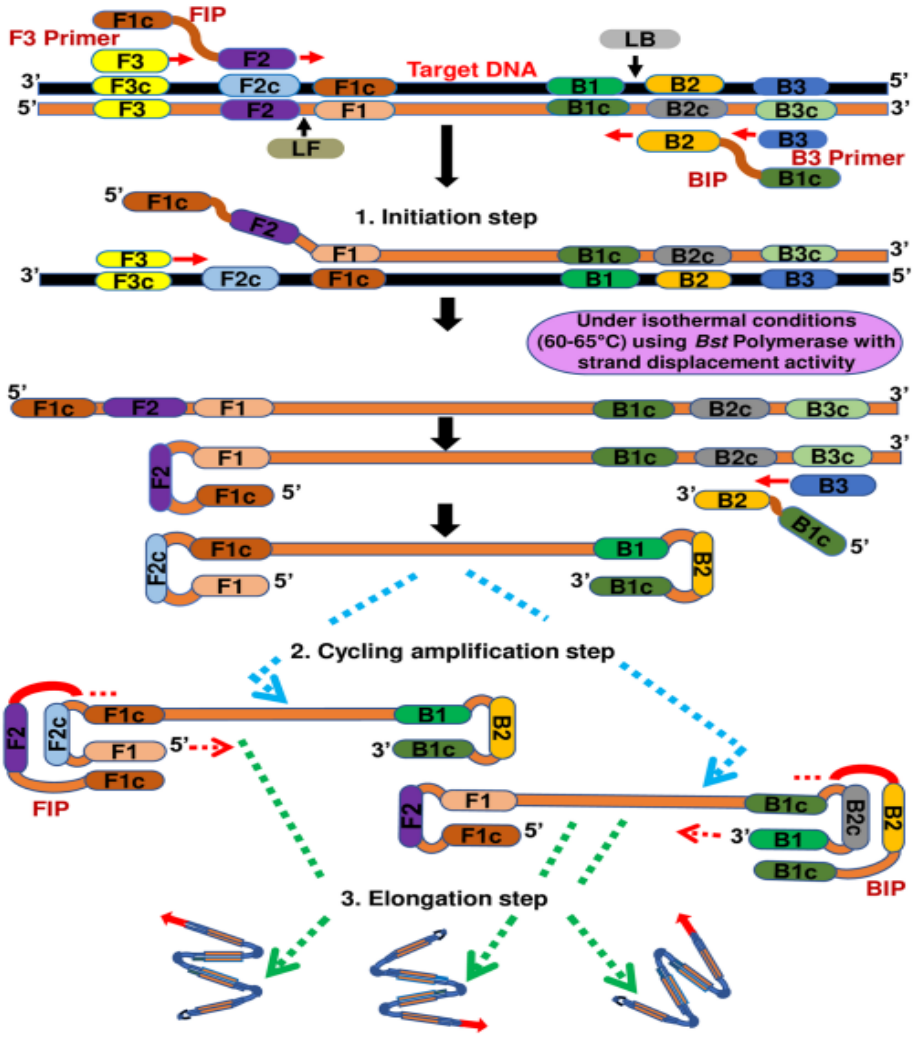

Figure 5: Stages involved in LAMP reaction process [24].

2.4.3 NASBA (Nucleic acid sequence-based amplification)

Nucleic acid sequence-based amplification (NASBA) is an mRNA-based technology. Measuring mRNA can confirm the presence and viability of a target organism. Because NASBA exclusively amplifies RNA, the presence of DNA in samples will not produce false positives. The complete amplification reaction is carried out at 41°C, which is the predefined temperature. The

homogeneous isothermal reaction requires three enzymes: reverse transcriptase (RT), RNase H,T7 RNA polymerase and two primers [25].

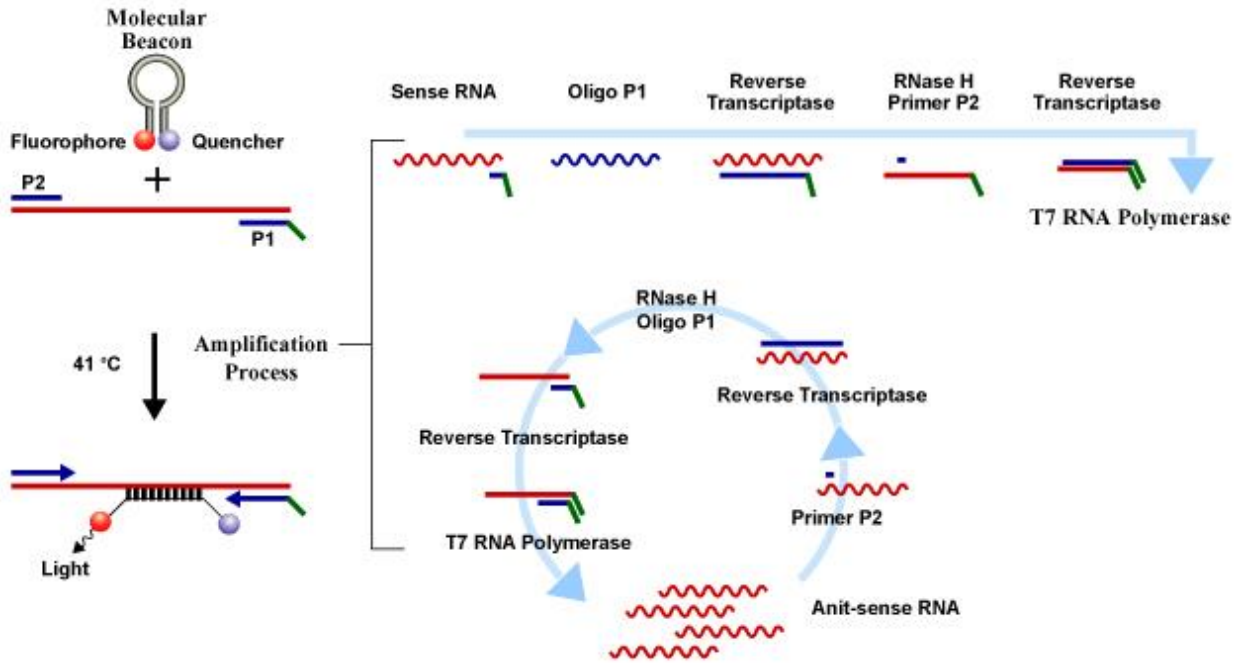

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Figure 6: stages of the NASBA amplification [26].

Figure6 shows the mechanism of reaction takes place during NASBA process. Primer P1 contains a 3' terminal sequence that is complementary a sequence on the analyte nucleic acid and 5' terminal sequence, that is recognized by T7 RNA polymerase. Primer P2 contains a sequence complementary to the P1 primer DNA strand. Addition of RNA initiates the NASBA process. A cDNA is synthesized by extending P1 with RT. RNA analytes are degraded by RNase H to allow P2 to anneal to single-stranded cDNA. When P2 is annealed to cDNA and extended with RT, it forms a DNA template for transcription and a double-stranded T7 promoter. In response, T7 RNA polymerase generates multiple copies of an RNA product. After that, each newly synthesized RNA product may anneal to P2, which a DNA product is synthesized with RT. DNA hybrid is degraded by RNase H, to allow the annealing of P1 to the 3' end of the DNA product. On annealing of P1, the DNA product is extended with RT using P1 as the template to form a double-stranded T7

promoter. The DNA product, now with a completed T7 promoter, directs the synthesis of many copies of the RNA product. Each newly transcribed RNA product can be used as a template for synthesis of a DNA product, which can be used as template for the further transcription of RNA product. The continuous repetition of this process results in the exponential synthesis of RNA and DNA products. A specific nucleic acid sequence of a DNA analyte may also be amplified by NASBA.

2.5 Software

Different kind of software are used for designing the primers and probe. Cross homology has been checked for the designed primers to make a specific and unique primer for the given sample. For designing PCR primers and probe, the genes were selected manually from a specified region and the NCBI website were used for creation and identifying the cross homology of the primers designed.

LAMP Designer is one of the software by premier Biosoft which is used in this thesis to designs efficient primers for Loop-Mediated Isothermal Amplification assays, that amplify DNA and RNA sequences at isothermal conditions, eliminating the necessity of a PCR setup. LAMP designer automatically interprets BLAST search results and avoids those regions to design primers that have significant cross homologies with the database. The primers can be BLAST searched against a database to verify their specificity.

Beacon DesignerTM is another software used to design NASBA primers and probe. Molecular biologists around the world use it to design successful real-time PCR assays. This saves time and money spent on failed experiments.

StepOne Software v2.3 is used for PCR and LAMP amplification and detection of DNA. BioTek Gene5 software provides endpoint, kinetic, spectral, and well area scanning for dual-mode and single-mode microplate readers.

3.0 Materials and Methods

In this chapter, the DNA/RNA extraction procedure by booms technology, selection of the primers and probes for the amplification and detection methods, and testing and development of the biomarkers with the three methods PCR, LAMP and NASBA has been explained. Initially, we started with the biomarker mito-CytB. This is a well-known biomarker with well-known PCR primers and probes to be able to detect eDNA from Esox Lucius. LAMP and NASBA primers and probes were therefore made using the software. We have also designed primers and probes from two more genes named as Histone and Glyceraldehyde 3-phosphate dehydrogenase. Experiments were performed on these biomarkers in the extracted samples with three methods PCR, LAMP and NASBA. Before the extraction procedure, the tissue and bone marrow raw samples to be collected from the fish named "PIKE". Some of the fishes were captured from the lakes in Horten at Norway which shown in figure 7.

Figure 7: Examples of the captured fishes from Norway.

3.1 Extraction of DNA/RNA by Booms Extraction

The protocol used for the extraction of Nucleic acids (DNA and RNA) was based on Boom extraction and the NucliSens easy Mag kit which shown in figure 8. The sample was treated with lysis buffer, silica beads, wash buffer and elution buffer.

Figure 8: Nuclisens Magnetic Extraction Kit (BioMérieux, FRANCE)

In this process to make the extraction to be good, the sample is added to the lysis buffer and Micro pestles were used to homogenize the sample in the solution. The amount of the cells was high in the homogenised sample as it is more viscous. Figure 9 shows the examples of the micro pestle used smashing the specimen.

Figure 9: Micro pestles used for homogenizing the sample (Bel-art, U.S)

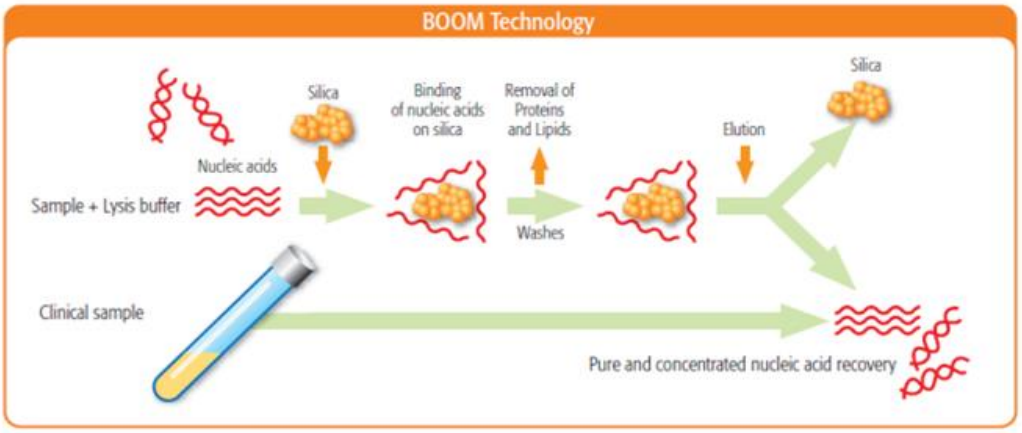

Figure 10: Booms extraction method for extraction of DNA\RNA [28].

The whole process in booms technology is shown in figure 10. All the buffers should be kept at room temperature for dissolving the crystals. It took 15 minutes to the crystals to be dissolved in a cold buffer. 500 µl lysis buffer was taken in a microcentrifuge tube and the sample from the pike were added to the same tubes. Micro pestles were used to homogenize the sample in the solution. The solution was incubated at room temperature for 10 minutes. Before adding silica beads to the sample, it has vortexed till it become an opaque solution. Then, the sample solution was added with silica beads. So, the tube was filled with sample, lysis buffer and silica beads. This mix was incubated at room temperature for the binding of DNA particles with the beads. After 5 minutes, the tubes were placed in a Mini Mag shown in figure11 and magnets were turned ON, so that the beads are allowed to settle towards the back of the tube.

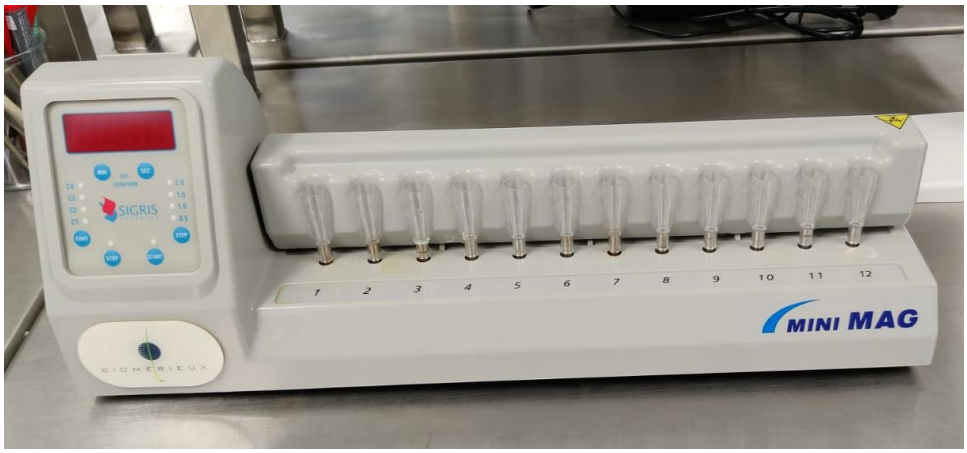

Figure 11: Magnetic rack (Mini MAG)

The supernatant is removed from the tube and 400 µl wash buffer 1 is transferred to the microtubes. Then the magnet was turned off and the solution was centrifuged for 10 seconds. After that the magnets are turned ON and wash buffer1 was removed from the tubes. The process of washing of the beads continues three times more with wash buffer 2, wash buffer3 and wash buffer 4 as 500 µl,400 µl and 400 µl respectively. Finally, the elution buffer was added to the tube for the unbinding DNA from the magnetic beads. The solution was incubated at 60°C in shaker for 5 minutes at a speed of 1400rpm. After elution, the tube was placed in magnetic rack and the transferred extracted nucleic acids was stored at -20°C in sterile microtubes [29].

3.2 Selection of the gene

An initial step in this process involved was the selection of a nucleotide sequence from the species Esox Lucius. Data was retrieved from NCBI's Nucleotide collection. The expression rate of the genome in different areas of sample was an important factor in choosing the specific genome. As there are no data on gene expression levels of northern pike, the approach was to find a housekeeping gene, which has a high and constant expression rate throughout the organism. A second important criterion was the specificity of the primers, in addition to the expression rate. The NCBI blast tool was used to align various mRNA sequences across species. It compares an input sequence to a database of selected sequences and shows an alignment of these sequences. In a blast search, the most important results to look at are query coverage, which indicates how much of the input sequence aligned with a found match, and percent identity, which indicates how well

the sequence aligned. The specific species of fish are checked only with other vertebrates in blast search since the specificity of the sample is checked only with vertebrates. In this experiment only selected genes were with the smallest cross homology.

3.3 PCR biomarkers development and testing

PCR primers and probes can be designed in many ways. Manual selection is one of the methods used in this process is to identify a specific region of a gene of interest. A specific primer was selected, and a primer blast was performed at NCBI to verify parameters of the designed primers. In addition to manual selection, NCBI and beacon designer were used for designing and verifying the PCR primers. Primers can either be checked in NCBI or in a software package called Beacon designer. Using this software, the desired probe can be created for each primer. Primers and probes on mitochondrial DNA were developed by the company NIVA for use in the eDNAuto project by using laboratory-developed primers and probes. Primers for the PCR were selected from the specific gene area and each primer was tested to see if it binds to the core sequence. Furthermore, it provides the melting temperature and GC content value of the primers, both of which affect the primer's binding properties. The TaqMan primers were also designed using beacon designer, which provides a higher specificity of binding with the sequence. An overview of how 10X master mix for histone and GAPDH prepared is shown in table 1 and 2. The formula used for preparing stock solution is V1C1=V2C2, where V1 is the known volume of stock, C1 is the stock concentration, C2 is the new concentration and V2 is the new concentration required for the solution.

Template name	Primer name	Yield (nmol)	Vol for 100µM(µl)	Final conc. in rx(µM)	Conc. in 10x (µM)	Total Vol. 10x Stock(µl)	Vol100µM for 10x(µl)	Again in main stock(µl)	dH2O (µl)
ElH2B	El_H2B_F	53.6	536	0.4	4	200	8	528	180
	El_H2B_R	50.8	508	0.4	4		8	500	
	NASBA_EL_MB	11.9	119	0.2	2		4	115	

Table 1: PCR concentration mixing volume for histone gene

Template name	Primer name	Yield (nmol)	Vol for 100µM(µl)	Final conc. in rx(µM)	Conc. in 10x(µM)	Total Vol. 10x Stock(µl)	Vol 100µM for 10x(µl)	Again in main stock(µl)	dH2O (µl)
EL_ GAPDH	EL_GAPDH_ F	37.1	371	0.4	4	200	8	363	180
	EL_GAPDH_ R	47.3	473	0.4	4		8	465	
	EL_GAPDH_ PROBE	17.7	119	0.2	2		4	115	

Table 2: PCR Concentration mixing volume of GAPDH gene

3.3.1 Protocol for PCR preparation

Before the experiments, it was very essential to clean the preparation bench, pipettes and the gloves sprayed with ethanol to create a contamination less environment. For the preparation of the master mix, the reagents must be kept at room temperature for melting to liquid form. The reagents were then moved to ice to prevent denaturation or any form of unwanted reactions. The reaction master mix was prepared in desired volume given in the table 3 [30]. An excess of reaction mix was made to compensate for dispensing losses (e.g., a 33-reaction mix for 30 reactions).

Component	Volume (µl)
Takyon ™ Master Mix	10
Forward primer	2
Reverse primer	2
Probe	2
Water	1 .5
Total Mix / reaction	17.5µl

Table 3: Reagents volume for the Reaction PCR master mix [31].

The reaction mix was transferred to the microtiter plate according to the number of samples to be tested. After reaction mix is filled, 2.5 µl of template DNA\cDNA or 2.5 µl of control DNA and

2.5 µl of water as negative control was pipetted to the same plate according to the kind of experiments. The bubbles inside the reaction well were removed with pipette tip. Finally, the microtiter plate was sealed with sealer cover and loaded into the Step One plus machine for the reaction.

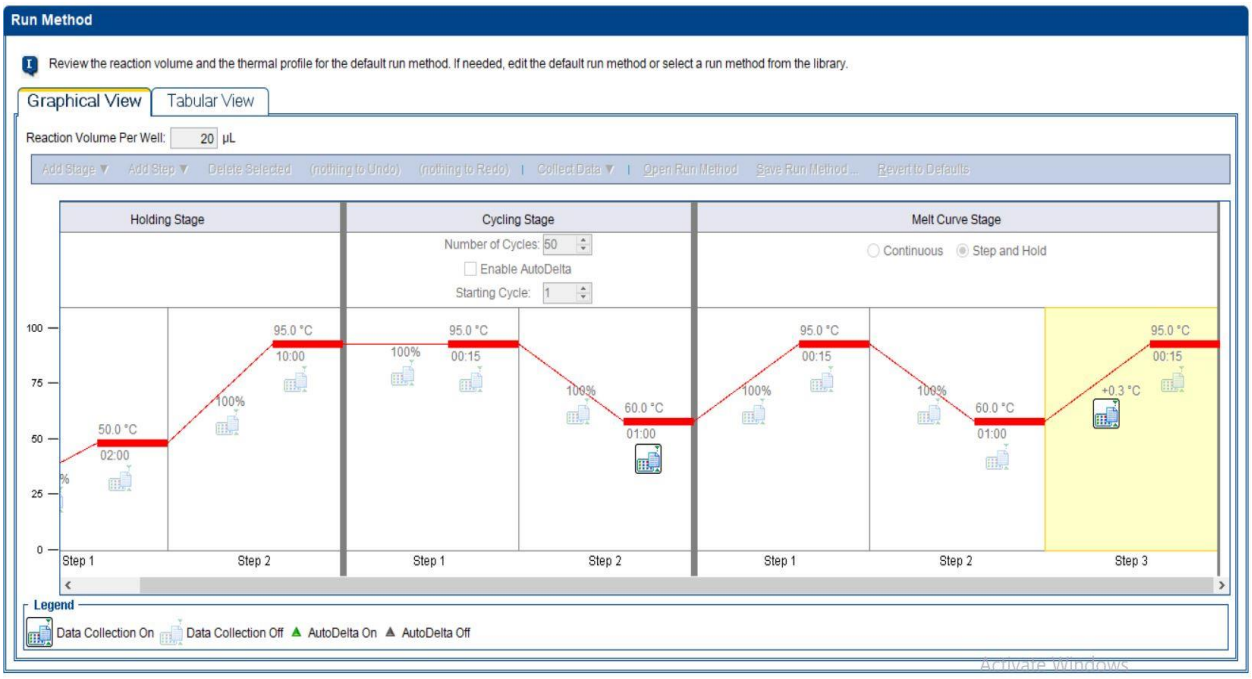

Figure 12: Example of graphical view of PCR reaction setup in StepOne instrument.

The figure 12 shows the reaction setup done for the PCR. It was the default setup used, but the number of cycles was changed according to expression rate or amplification of the different genes. The PCR was done on three different genes (mitochondria and the selected genes). Different experiments were carried out on these genes.

3.3.2 PCR on tissue and bone marrow

Different tissue samples were extracted and 1:10 dilutions were used for the PCR test on mitochondria sample. These tissue samples are treated with designed primers of mitochondria cyt b. The samples were named as E, F, G and H. Pure samples E, F and G were taken for the histone gene and For GAPDH new sample were extracted from the same fish and named as L, M, N and O. Figure13 shows an example how to setup the plate layout for a PCR reaction.

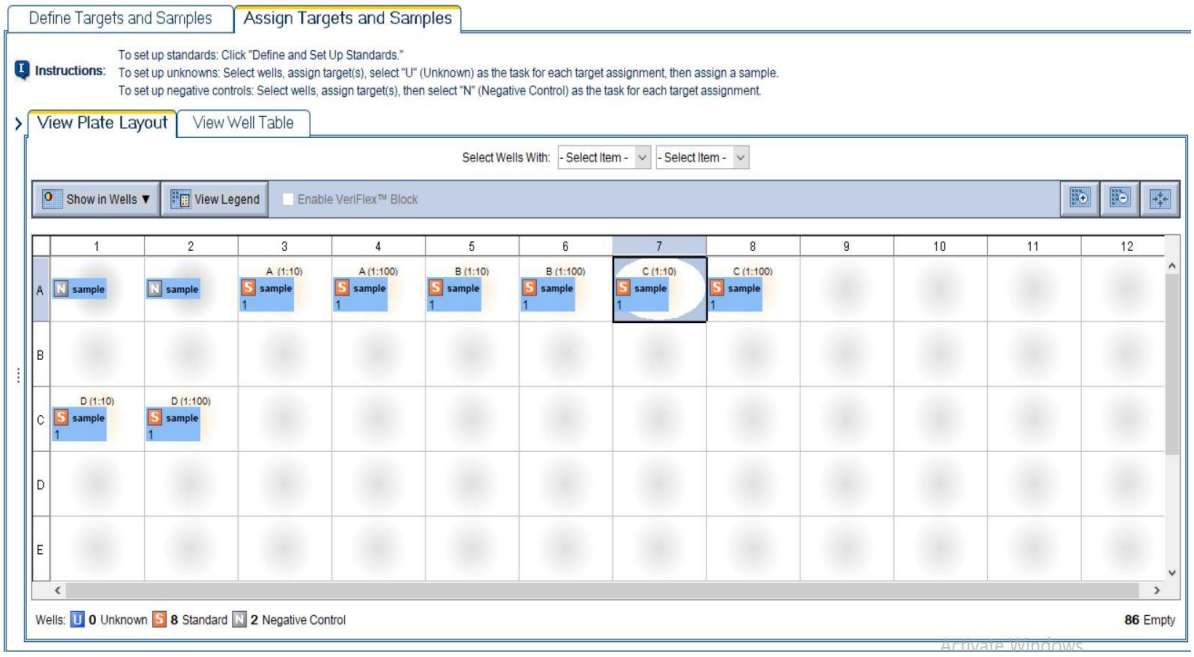

Figure13: Example of a Plate layout setup for PCR reaction.

Before loading the samples to the machine, the plate layout has been set according to the figure. Along with the samples two negative samples also been added to verify the results of the samples are true positives. Similarly, the same procedure was done on the selected genes.

Two samples from bone marrow were used for the experimental procedure. Each sample has a dilution of 1:1 and 1:10 were made for mitochondria cyt b gene. Bone marrow samples were also tested against the primers created for histone and GAPDH. The PCR reaction was carried out on mitochondria cyt b, histone H2B, and GAPDH mRNA genes and the amplification curves were observed.

3.4 LAMP Biomarkers: Designing and Testing

All primers used in this study were designed by LAMP Designer 1.12 and procured from Euro fin. The primers were reconstituted according to the required concentration. The primers are designed so that the distance from the end of F2 to the end of B2 is between 120 bases and 160 bases. The primers are also designed so that the distance from the 5' end of F2 to the 5' end of F1 is between 40 bases and 60 bases. The specific region for the LAMP primers were selected according to the same location of the PCR and NASBA primers in Histone and GAPDH gene.

3.4.1 Protocol for LAMP preparation:

The warm start LAMP Kit was employed for the reaction setup, throughout the research and the components in the kit were Warm Start, LAMP 2X Master Mix and LAMP Fluorescent Dye. The master mix is expected to contain dNTPs mix, *Bst* DNA polymerase, Tris HCl, KCl, Tween, $(NH_4)2SO_4$, betaine, MgSO4, and fluorescent dye could be FAM/SYBR Green. The reaction was carried out in 25 µL volume, as suggested by the warm start manual. The reaction setup is listed in Table 4.

Component	Volume in µL	
	Target Detection	Control
Warm Start LAMP 2X Master Mix	12.5	12.5
Fluorescent dye (50X)	0.5	0.5
LAMP Primer Mix (10X)	2.5	2.5
Target DNA/RNA	1	-
Dis. Water	8.5	9.5
Total	25	25

Table 4: Reagents volume for the Reaction LAMP master mix[32]

Generally, master mix was prepared without target and 24 µL was transferred to necessary number of wells. Then,1 µL of target DNA was added to the well. Finally, all the components are thoroughly mixed and sealed. The microtiter plate holding sample was placed in the instrument with the incubation temperature of 65 ºC, for real time detection. The default reaction setup for a lamp reaction is shown in figure14.

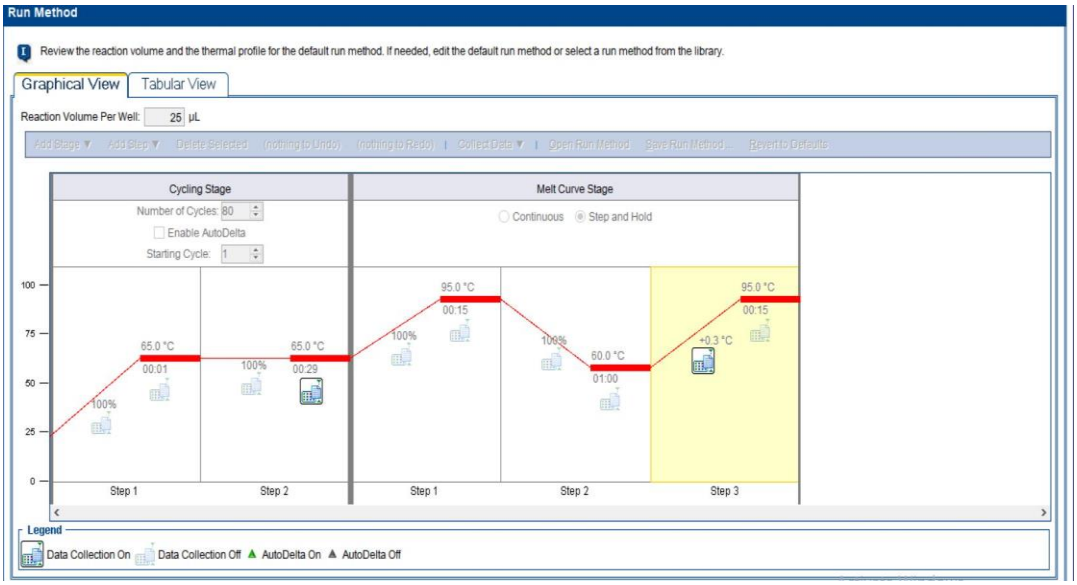

Figure 14: Example of graphical view of PCR reaction setup in StepOne instrument

3.4.2 LAMP on Tissue and bone marrow

Similar samples that were used in PCR reaction is used for the LAMP reaction. Sample E, F, G and H are used for mitochondrial cyt b primers and HISTONE. Negative controls are also used to verify the results for each reaction. Reaction mix for the LAMP on tissue on all three gene were prepared according to the protocol. Tissue samples and bone marrow samples were used for testing the biomarkers of all three genes for LAMP reaction experiment.

3.5 NASBA Biomarker Development and Testing

All primers used in this study were designed by BEACON DESIGNER. There are so many assays, to design different kind of primers. For designing the NASBA primers and probe we need to select the Beacon assay. The position of the primer sequence is selected according to the availability of the molecular beacon. Two NASBA primers and probes from different regions of the genomic sequence are created for GAPDH to check both the best one. The factors such as Length of the primers, melting temperature and GC content are carefully considered during the design of the primers and probes. The designed primers were ordered from eurofin. The primers were reconstituted according to the required concentration.

3.5.1 Protocol for NASBA preparation

The NASBA kit consist of Reagent sphere and Enzyme sphere which are in solid form which were diluted with 80 µl reagent diluent and 57 µl enzyme diluent respectively. The enzyme diluent with solid is kept 20 minutes in room temperature to dissolve the mix. Each NASBA kit is used for the preparation of 10 samples. The components for making NASBA master mix for 10 samples is given in table 5.

Component	Volume (µl)
Reagent mix	80 µl
Primer mix	10 µl
probe	2.5 µl
KCL	16 µl
NASBA water	11.5 µl

Table 5: NASBA master mix components for 10 samples [33].

The Prepared master mix is distributed onto PCR plate/strips at room temperature.10 µl master mix,5 µl sample and 5 µl enzyme mix is added to each well. NASBA water is added as negative control for each experiment. The Reader was preheated, and sample is incubated to 41°C.After incubation the plate is placed on a heating block keeping 41 °C. It is then inserted to the bio-Tek synergy machine for the reaction.

3.5.2 NASBA on Tissue and bone marrow

Four samples from esox lucius were named as A1, A2,A3 and A4 are taken as test samples for the tissue against the mito cytb primers and probes and two negative samples A5,A6 were also used for the experiment. These four pure samples are diluted 1:10 ratio and named as C1,C2,C3and C4 which were used for testing Histone primers and probes against the Tissue samples. Reaction mix for the NASBA on tissue on all three gene were prepared according to the protocol. Bone marrow samples were also used for testing the biomarkers of all three genes for NASBA reaction experiment.

3.6 Efficiency, Sensitivity and Specificity

For Efficiency and sensitivity, A series dilution study of sample was conducted. The pure sample was diluted up to 10^5 dilutions. A total of three different extracted tissue samples named as E, F and G from species were used for the experiment. The dilutions were made as follows. Total of five micro tubes were taken and marked as 1:10,10^2,10^3,10^4 and 10^5. All the tubes were filled by 90 µl of water. 10 µl of sample was added to the first tube (i.e,10^2) and the solution was vortexed for 10 sec. From the first tube, 10 µl of solution was taken and added to the second tube (i.e,10^2).This process was continued till all the tubes were filled. So, the desired dilution with one replicate for each was made for the experiment.

For specificity test, eight different fish species are collected which were different types and the samples are extracted from each of them. The specificity of the test was done with these fishes along with pike sample and negative controls. PCR primers of different genes were tested against these fishes for testing the specificity of the sample.

3.7 Oligos

The Oligos contain long base pairs that are designated in such a way that they contain all primers and probes that are needed for PCR, LAMP, and NASBA. Oligo with 308 base pair is selected for HISTONE gene and an oligo with 411 base pair is selected for GAPDH. Oligoes must contain all the exact same sequence of the target gene that is to be amplified. Dilutions of 1:1,1:10,1:10^2,10^3,10^4,10^5,10^6 are made for PCR and LAMP reaction. Two negative samples were also added to confirm the result. Oligos were tested against PCR, LAMP and NASBA primers and probes to verify the designed primers and probes from each genome is working well.

4.0 Result

4.1 Samples collection

The extracted tissue, bone marrow from Esox *Lucius* was subjected to dilutions. A total number of 26 samples was extracted for the experiments. The samples extracted was named as E, F, G, H, L, M, N and O and other fish species as F1, F2, F3, F4, F5, F6, F7 and F8.

4.2 Design of primers and probe for PCR, LAMP and NASBA

PCR primers and probes for the mitochondria cytb in table 6 was procured from a Norwegian company called NIVA while LAMP and NASBA primers and probes of mitochondria cyt b in table 7 and 8 were constructed by LAB faculty and supervisor in our Laboratory. The forward and reverse primers are shown in table 6 is used for binding the original sequence.

Gene	Type	Sequence
Mitochondria cyt b	Forward primer	CTCCACAGCCTTCTCATCAGTCT
	Reverse primer	TTCGGATAAGTCAGCCGTAGTTAA
	probe	CCACATCTGCCGGGAC

Table 6: Mito cytb PCR primers and probes

Gene	Type	sequence
Mitochondria cytb	Forward outer primer-F3	TTCAAGGCACCTGTATTGG
	Backward outer primer-B3	ACTGTAACTAAGAAAGAGCC
	Forward inner primer-FIP(F1C+F2)	TCGTCATTATTCCCCGGCAAA ATGGCCTCTAAACCCATTG
	Backwaard inner primer-BIP(B1C+B2)	AGTCGGCACAGCCTTAAGCCT GGTCGTCACCTAAGAGA
	Loop primers F	ATCAGCGTGTGATTGCCA

	Loop primers B	CCGAACTAAGCCAGCCAG

Table7: Mito cytb LAMP primers and probes

Gene	Type	Sequence	Position	Length	TM
Mitochondria cyt b	Sense primers	TTTATTTAGTATTTGGTGCTTGA	5 492	23	53.5
	Anti-sense primers	CCTGGCTGGCTTAGTTCGGC	5 554	20	63.9
	Molecular beacon [2]	CGCGATGCCGAACTAAGCCAGCC AGGATCGCG	61,64		61.64

Table8: Mito cytb NASBA primers and molecular beacons.

The Complete genomic FASTA sequence for Esox Lucius histone h2b is shown below. The location of the designed primers is shown in the below h2b sequences.

4.3 Selection of the gene

In most of the times, housekeeping genes are anticipated to express at constant level, because it involves in basic cell maintenance. Herein, Mitochondria Cytochrome B (Cyt B), Histone(H2B), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene of *Esox Lucius* were considered with main criteria of cross homology and query value percentage. H2B, GAPDH primers and probes were designed by us and described in detail, whereas Cyt B was procured.

4.3.1 Esox Lucius histone H2B-like (LOC105027228), mRNA

NCBI Reference Sequence: NM_001310976.1

GenBank Graphics

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>NM_001310976.1 Esox Lucius histone H2B-like (LOC105027228), mRNA
GGGGGGCATTCATTTTTGAGGATCATCAGGAAATCATCATGCCCGAACCAGCAAAGTCCGCCCCTAAGAA
GGGCTCTAAAAAAGCCGTCACCAAGACGGTAGGCAAGGGTGGTAAGACGCGCAGGAAATCCAGGAAGGAG
```

[2] Molecular beacons are single stranded hairpin shaped oligonucleotide probes[4].

AGCTATGCCATCTACGTGTACAAAGTCCTGAAGCAGGTTCACCCGGACACCGGTATCTCCTCCAAAGCCA
TGGGAATCATGAACTCATTCGTGAGCGACATCTTCGAACGTATCGCGGGGGAGTCATCTCGTCTGGCCCA
CTACAACAAGCGATCTACCATCACCTCGAGAGAGATTCAGACCGCCGTGCGGCTGCTGCTCCCCGGTGAG
CTGGCCAAACATGCCGTGTCCGAGGGGACTAAGGCCGTGACCAAGTACACCAGCTCTAAGTAAACCGCTA
CTGTACTTCCAAGGGTCCAATTACCCCAAAGGCTCTTTTGAGAGCCACTCACTGTGTGGGTATAAAAGCA
ATTCGGAGTTGAACGAATTAACAAAATATGTTGTATTCTATAGTAGCCGCACGCTGTCGACTGATAACAT
ATGAAATAAACGGCGTACAGCCGAGTGCATACATTGTATTAACTAAACTATTAACTGTGGCTATATGTTT
ATAAAGCAGCTAGCGAGAGGGATACAATGAATCCTTTCACCAGTTCCTGTTTGAATCGTGAGCGTTGCAG
GACCAGTATGTGGGGCTTTGCTCGCCAATTTTTTTTGTGTTAACTGATTGTACCACGCCTTTGAAAGCCA
TGACTCGCAATGTCTCTCAATCAAGTCACGAGAGTGCGGCTTTAGTGAACGTTGAAATAAATACCACATG
ACGG.

Standard, criteria was followed in designing primers and probes of the chosen gene. Along with the primer sequence, exported condition such as length, start and stop point, melting temperature (Tm), GC percentage was given in the respective tables. Respective Colors used in the table 9,10 and 11 are used to represent selected primers in the whole sequence.

NAME	**Type**	Sequence (5'->3')	Length	Start	Stop	Tm	GC%
HISTONE (H2B)	Forward primer	TCTATAGTAGCCGCACGCTG	20	527	546	59.41	55
	Reverse primer	TCACTAAAGCCGCACTCTCG	20	818	799	60.11	55
	Probe/Molecular beacon (MB)	CGCGATGAGCGTTGCAGGACCAG TATGTATCGCG	34	654	688	60.03	
	Oligoes	TATTCTATAGTAGCCGCACGCTGT CGACTGATAACATATGAAATAAA CGGCGTACAGCCGAGTGCATACA TTGTATTAACTAAACTATTAACTG TGGCTATATGTTTATAAAGCAGC TAGCGAGAGGGATACAATGAAT CCTTTCACCAGTTCCTGTTTGAAT CGTGAGCGTTGCAGGACCAGTAT GTGGGGCTTTGCTCGCCAATTTTT TTTGTGTTAACTGATTGTACCACG CCTTTGAAAGCCATGACTCGCATG TCTCTCAATCAAGTCACGAGAGTG CGGCTTTAGTGAACGTTGAAATAA	308	524	830		

Table9:H2B PCR primers and probe

Gene	Type	Sequence	POSITION	LENGTH	TM	GC%
Histone (H2B)	Forward outer primer-F3	GCCGAGTGCATACATTGTA	580	19	60	47.4
	Backward outer primer-B3	TTTCAACGTTCACTAAAGCC	827	20	58.9	40
	Forward inner primer-FIP(F1C+F2)	CATACTGGTCCTGCAACGCTCG AGAGGGATACAATGAATCC		40		
	Backwaard inner primer-BIP(B1C+B2)	ACTGATTGTACCACGCCTTTGA GCACTCTCGTGACTTGATT		40		
	Loop primers F	CGATTCAAACAGGAACTGGTG	688	21	61.8	47.6
	Loop primers B	AGCCATGACTCGCAATGT	766	18	61.8	50

Table10: H2B LAMP primers

Gene	Type	Sequence	Position	Length	TM	GC%
Histone (H2B)	Sense primers	CTAAACTATTAACTGTGGCTA	603	21	51	33.3
	Anti-sense primers	AATTCTAATACGACTCACTATAGGGGGTAC AATCAGTTAACACAA	754	20	51.4	35

Table 11: H2B NASBA primers and probes

The whole genomic sequence of the GAPDH used for the designing of the primers and probes are shown below and table 12,13 and 14 represent PCR,LAMP and NASBA primers and probes respectively.

4.3.2 Esox Lucius glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mRNA

NCBI Reference Sequence: XM_010884975.4

GenBank Graphics

>XM_010884975.4 PREDICTED: Esox lucius glyceraldehyde-3-phosphate dehydrogenase (gapdh), mRNA

CAACCCTGCAAGTCCCCACTCACCAGAACGTCGCAAGACAGGACTGAGGCATCTCACAAACGAGGACTAA
ACCAACTCAGGCAAAATGGTGAAAGTCGGTGTTAATGGATTTGGCCGTATCGGGCGCCTGGTGACTCGTG
CTTCTTTCCAATCCAAGAAAGGAGTAGAGGTTGTGGCCATCAATGATCCCTTCATTGACCTGGACTACAT
GGTCTACATGTTCAAGTATGACTCCACCCACGGACGTTTCCATGGTGAGGTAAAGGCCGAGGGTGGCAAG
TTGGTCATCGATGGACACGCCATCACAGTGTTCCACGAGAGAGACCCAGCAAACATCAAGTGGGGAGATG
CTGGCGCCACCTATGTGGTTGAATCAACAGGTGTATTCACCACCATTGAAAAGGCCTCCGCCCATCTAAA
GGGAGGTGCCAAGAGGGTCATCATCTCTGCTCCCAGCGCTGATGCACCCATGTTTGTCATGGGTGTCAAC
CACGAGAAGTACGATAACTCCCTCAAGGTTGTCAGCAATGCTTCATGCACAACCAACTGCCTGGCTCCCC
TGGCCAAGGTCATCCATGACAACTTCCACATCATTGAGGGTTTGATGAGCACTGTCCACGCCGTCACCGC
CACCCAGAAGACCGTTGATGGTCCCTCTGGAAAGCTGTGGAGGGATGGACGCGGCGCCAGCCAGAACATC
ATCCCCGCCTCCACCGGAGCTGCCAAGGCTGTCGGCAAAGTCATCCCAGACCTGAACGGCAAGATCACAG
GCATGGCTTTCCGTGTCCCCACCCCCAACGTTTCAGTGGTTGACCTGACTGTCCGCCTGGAGAAGGCTGC
CAGCTACGAAGACATCAAGAAAGTGGTCAAGGCTGCTGCTGATGGACCCATGAAGGGAATTCTTGGTTAC
ACAGAGGACCAAGTTGTGTCCACAGACTTCAACAGTGACATTCGCTCCTCCATCTTCGATGCTGGTGCTG
GAATTGCACTGAACGACCACTTTGTCAAGCTGGTTACATGGTACGACAATGAATTTGGCTACAGCAACCG
CGTCATTGACCTGATGGCTCACATGACCACCAAGGAGTAAAGCCAATCGACCAATCAAATGTCCCCGAAC
TACGCACGACCCATATACTTCCCATTAAATTGGACTCTAGCCCTCGACTTGCCTGAGAAAGTTAACCGCA
TAAAGGAAATCACAACTTGAA

Gene	Type	Sequence (5'->3')	Length	start	stop	Tm	GC%
GAPDH	Forward primer	CTTGGTTACACAGAGGAC	18	902	919	60.7	50
	Reverse primer	GTTGCTGTAGCCAAATTC	18	1048	1031	60.5	45
	Probe	CAACAGTGACATTCGCTCCTCC	25	940	964	70	52
	oligoes	TTTCAGTGGTTGACCTGACTGTC CGCCTGGAGAAGGCTGCCAGCT ACGAAGACATCAAGAAAGTGGTC AAGGCTGCTGCTGATGGACCCATGA AGGGAATTCTTGGTTACACA					

		GAGGACCAAGTTGTGTCCACA GACTTCAACAGTGACATTCGCT CCTCCATCTTCGATGCTGGTGC TGGAATTGCACTGAACGACCA CTTTGTCAAGCTGGTTACATGG TACGACAATGAATTTGGCTACA GCAACCGCGTCATTGACCTGATG GCTCACATGACCACCAAGGA GTAAAGCCAATCGACCAATCAA ATGTCCCCGAACTACGCACGACC CATATACTTCCCATTAAATTGGA CTCTAGCCCTCGACTTGCCTGAG AAAGTTAACCGCATAAAGGAAATC ACAACTTGAA					

Table12: GAPDH PCR primers and probe

Gene	Type	Sequence	POSITION	LENGTH	TM	GC%
GAPDH	Forward outer primer-F3	CAGAGGACCAAGTTGTGTC	912	19	60.1	52.6
	Backward outer primer-B3	CAAGTCGAGGGCTAGAGT	1171	18	60.1	55.6
	Forward inner primer-FIP(F1C+F2)	GCTTGACAAAGTGGTCGTTCAG ACATTCGCTCCTCCATCT		40		
	Backwaard inner primer-BIP(B1C+B2)	TACAGCAACCGCGTCATTGAGG TCGATTGGCTTTACTCC		39		
	Loop primers F	GCAATTCCAGCACCAGCATC	987	20	64.7	55
	Loop primers B	GATGGCTCACATGACCACCAA	1063	21	65.1	52.4

Table 13: GAPDH LAMP primers and probes

Gene	Type	Sequence	Position	Length	TM	GC%
GAPDH	Sense primers	ATTCTTGGTTACACAGAGGA	899	20	54	40
	Anti-sense primers	AATTCTAATACGACTCACTATAGGG AAATTGCTGTAGCCAAATTCATT	1047	20	54.1	35
	Molecular beacon	CGCGATAACAGTGACATTCGCTCC TCCAATCGCG	941	22	60.9	

Table 14: GAPDH NASBA primers and probes

4.4 PCR on Tissue

PCR was performed for Cyt B primers with extracted DNA, where the samples were labeled as E, F, G and H and diluted as 1:10 samples each. The amplification plot of Tissue sample against mito cytb sample is shown in the figure 15.

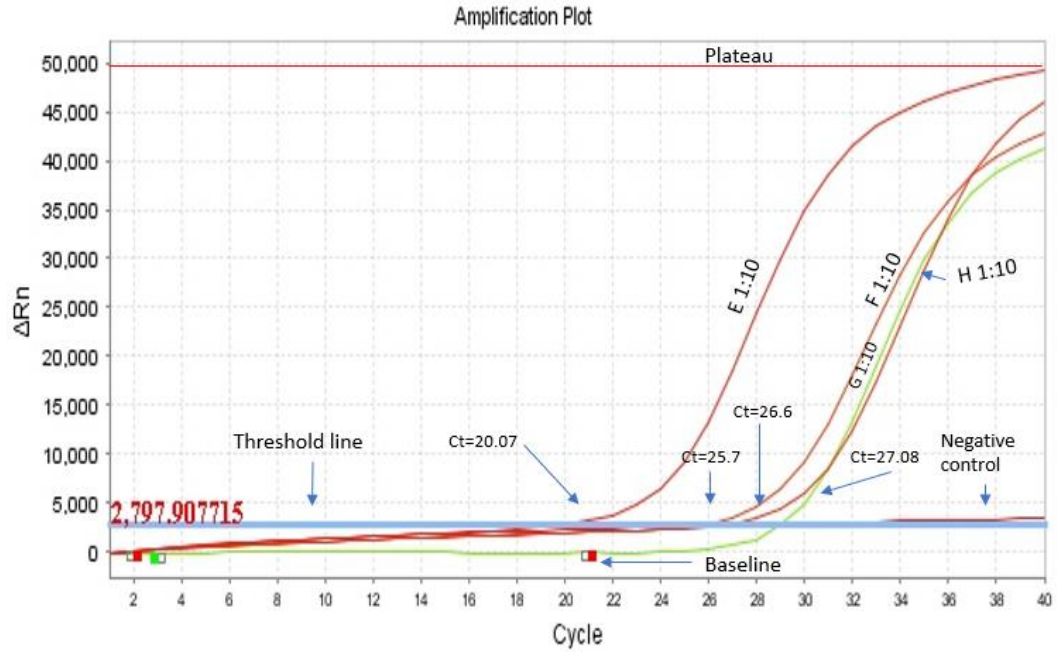

Figure 15: PCR Amplification plot by mitochondria cyt b primers on tissue

Delta Rn shows the relative fluorescence values on the x-axis and y-axis says the number of cycles of each reaction. The Ct values (That is, the cycle number at which the fluorescent signal of the reaction crosses the threshold) of the respective amplification plot is shown in figure15. Ct is the relative measure of the original concentration of the target in the PCR reaction. The baseline here is the measurement in PCR means the level of signal during the first few cycles. During this time, there is little change in fluorescence is noted, which means the signal detected establishes the level of background signal. The amplification plot reveals that designed cytb primers were successfully active towards Target. It also shows the accumulation of DNA templates over the complete duration of the PCR experiment. From the graph, a region called plateau is defined where the rate of accumulation of the DNA molecules is decreased. An example, how the Rn values of the amplification was extracted from the PCR machine is shown in Table 15.

Rn Values

Cycle	Target Name	negative	E 1:10	F 1:10	G 1:10	H 1:10
1	CytB	13674.8291	11693.8105	13496.3066	13990.474	11038.696
2	CytB	14047.0938	11994.6563	13654.3984	14170.009	11212.115
3	CytB	14189.5039	12219.248	13843.3965	14305.772	11304.72
4	CytB	14327.8398	12383.1543	14023.7051	14416.97	11424.857
5	CytB	14443.6211	12567.7813	14133.7578	14533.202	11537.375
6	CytB	14598.5215	12682.8838	14276.8975	14709.399	11709.684
7	CytB	14702.1533	12842.4824	14390.7822	14823.733	11718.322
8	CytB	14787.1758	12950.5918	14502.4209	14920.113	11848.011
9	CytB	14930.2783	13091.7676	14596.5156	15047.055	11920.275
10	CytB	15051.666	13199.5352	14661.6699	15145.327	12008.654
11	CytB	15166.3135	13347.4863	14753.8613	15252.745	12166.672
12	CytB	15279.4316	13452.3945	14854.9072	15376.781	12164.623
13	CytB	15362.0625	13554.9639	14970.5908	15434.948	12276.545
14	CytB	15448.6104	13689.334	15055.9141	15570.668	12337.223
15	CytB	15572.5918	13814.7715	15111.584	15652.868	12411.628
16	CytB	15645.4189	13950.2734	15195.0537	15745.067	12364.668
17	CytB	15719.4697	14085.1797	15281.665	15810.649	12455.533
18	CytB	15778.1543	14209.0645	15351.4365	15917.023	12512.289
19	CytB	15876.9688	14391.6855	15452.4092	15983.418	12611.795
20	CytB	15963.8145	14635.9482	15528.8516	16072.726	12703.268
21	CytB	16018.207	15012.1465	15611.8945	16131.668	12860.744
22	CytB	16049.5498	15619.2207	15666.9277	16226.469	12854.324
23	CytB	16110.1748	16595.666	15769.1797	16306.997	12875.373
24	CytB	16173.1328	18346.0938	15888.6738	16437.932	13052.207
25	CytB	16269.2891	21076.9922	16013.8027	16678.27	13220.697
26	CytB	16281.2227	25128.4961	16226.4209	16995.221	13464.777

27	CytB	16330.2695	30383.4297	16498.6113	17617.805	13949.746
28	CytB	16372.501	36176.4883	17047.1016	18658.348	14697.773
29	CytB	16462.7773	41724.9375	17954.1445	20428.43	16208.631
30	CytB	16474.9531	46642.5	19493.1641	23234.098	18492.51
31	CytB	16551.5684	50454.2344	22035.1777	27178.998	22178.008
32	CytB	16565.1367	53306.7813	25895.5273	32075.639	26968.852
33	CytB	16658.8691	55281.1875	30911.8574	37314.836	32804.844
34	CytB	16685.9023	56814.5742	36576.5352	42310.086	38510.457
35	CytB	16791.3711	57854.2969	42411.6328	46616.359	43799.953
36	CytB	16815.1875	58767.1523	47644.7969	49983.219	47568.203
37	CytB	16891.5859	59442.4688	52044.5	52598.512	50868.75
38	CytB	16936.8672	60063.1094	55301.168	54372.785	52841.426
39	CytB	16995.0977	60605.1797	57830.9063	55726.309	54424.164
40	CytB	17031.1094	61131.1367	59560.7969	56865.941	55535.441

Table 15: Fluorescence Values of Each PCR Mito cytb Tissue Samples

The above said three similar samples E, F and G were used to test the constructed H2B primers, and amplification plot is shown in the figure 16. The amplification starts at the point of 30th cycle.

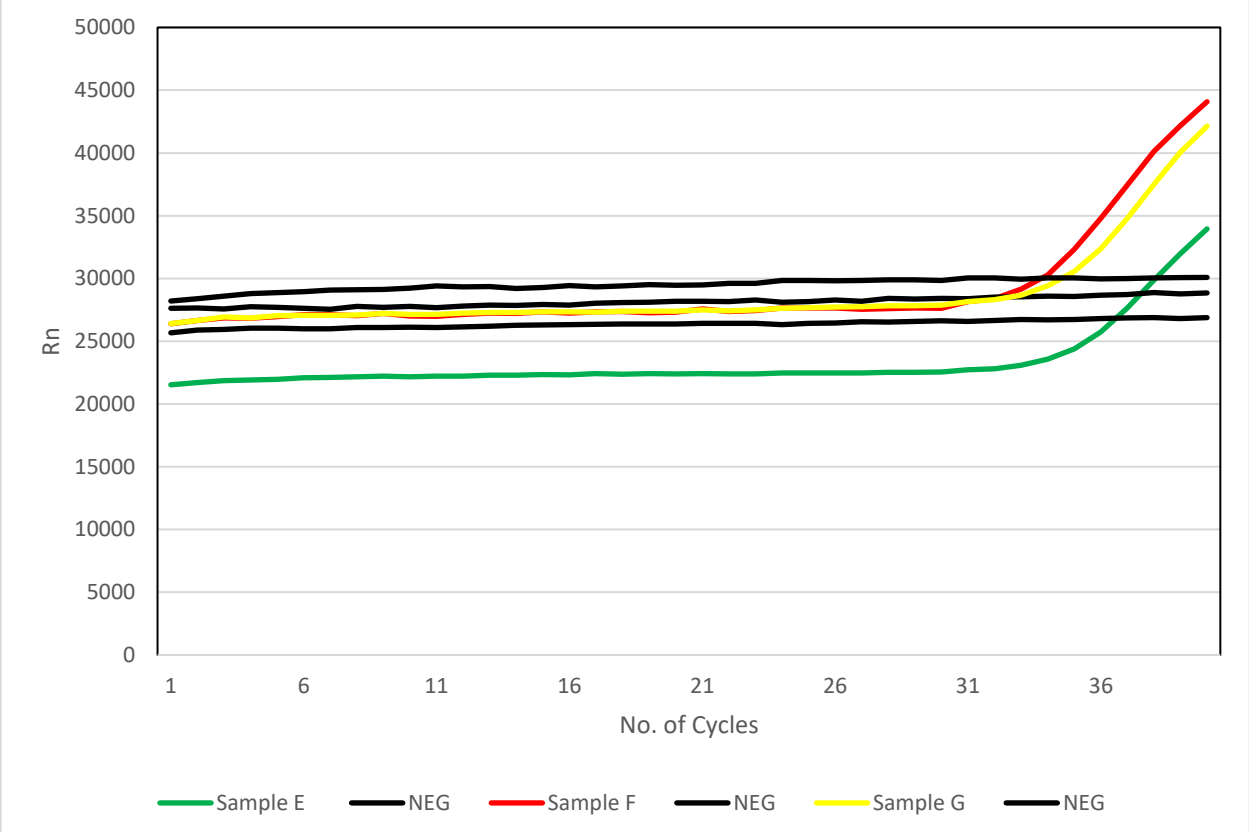

Figure 16: PCR amplification plot by Histone(h2b) primers on tissue.

The amplification by Histone primers in figure 16 tells us that the E, F and G has Ct values between 30-35. As, the amount of DNA template decrease, the Ct value increases. So, the whole reaction is positive but has a poor amplification signal. The graph is replotted in Microsoft excel by getting the amplification values from the PCR instrument. Here Normalized Rn value is taken as we can see that the cycle fluorescence signal starts between 2000 to 3000 range. It is evident from the amplification signal that the DNA template amount is not sufficient in the sample to get a good amplification.

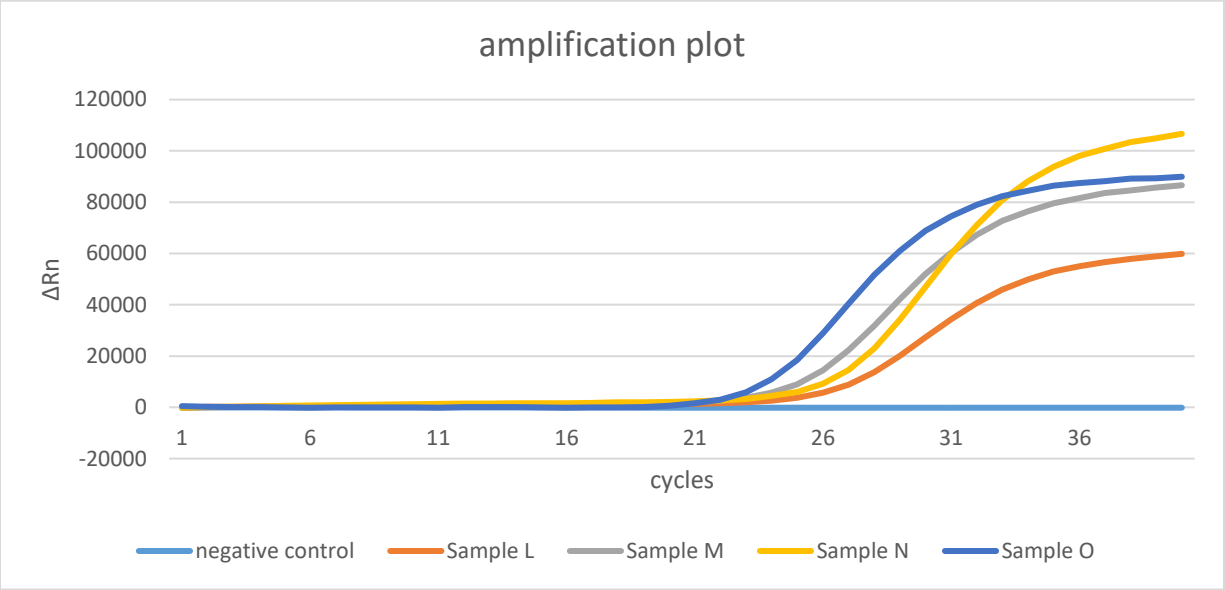

Figure 17: PCR Amplification by GAPDH on tissue

Esox Lucius, tissue sample, which was marked as L, M, N, O were made used in experiment with GAPDH primers, and the amplification cycle is shown in the figure 17. The exponential phase was observed at the cycle between 25 and 30 and confirmed as positive signal along with negative control.

Sample	Ct value
Sample L	22.5
Sample M	23.9

Sample N	24.7
Sample O	22.8

Table16: PCR Ct values of GAPDH, mRNA on Tissue

The Ct values in the figure is mentioned in the table 16 tells that the concentration of the target in the PCR reaction is high.Overall, the PCR primers and probes of the selected genes showed a good binding to the targeted Tissue samples of Esox Lucius.

4.4.1 PCR on Bone marrow

DNA obtained from bone marrow also used as template to experiment. The amplification plot towards Cyt B primers and H2B primers are shown in the figure 18 and figure 19, respectively. It evidenced that; genes were active against those designed primers. Comparatively, the fluorescence of reporter dye (Rn) is less for bone marrow cell to tissue. However, the amplification starts in the early cycle of 19 to 24 and shown in the figure 18. Similar range of amplification is seen in three sample of interest.

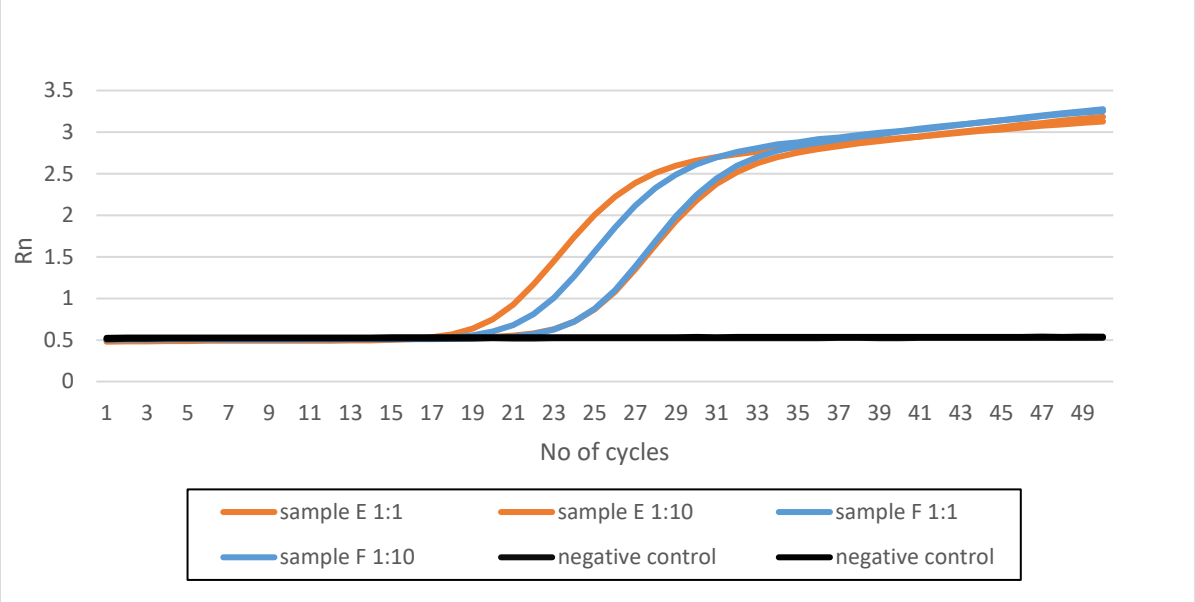

Figure 18: Amplification plot by PCR on mitochondrial cyt b on bone marrow

Histone primers are successful in the direction of amplifying the bone marrow template. Increased Rn value is observed, though the amplification starts at late stage of 26th cycle. Three set of sample exhibits the nearby pattern of amplification and shown in the figure 19.

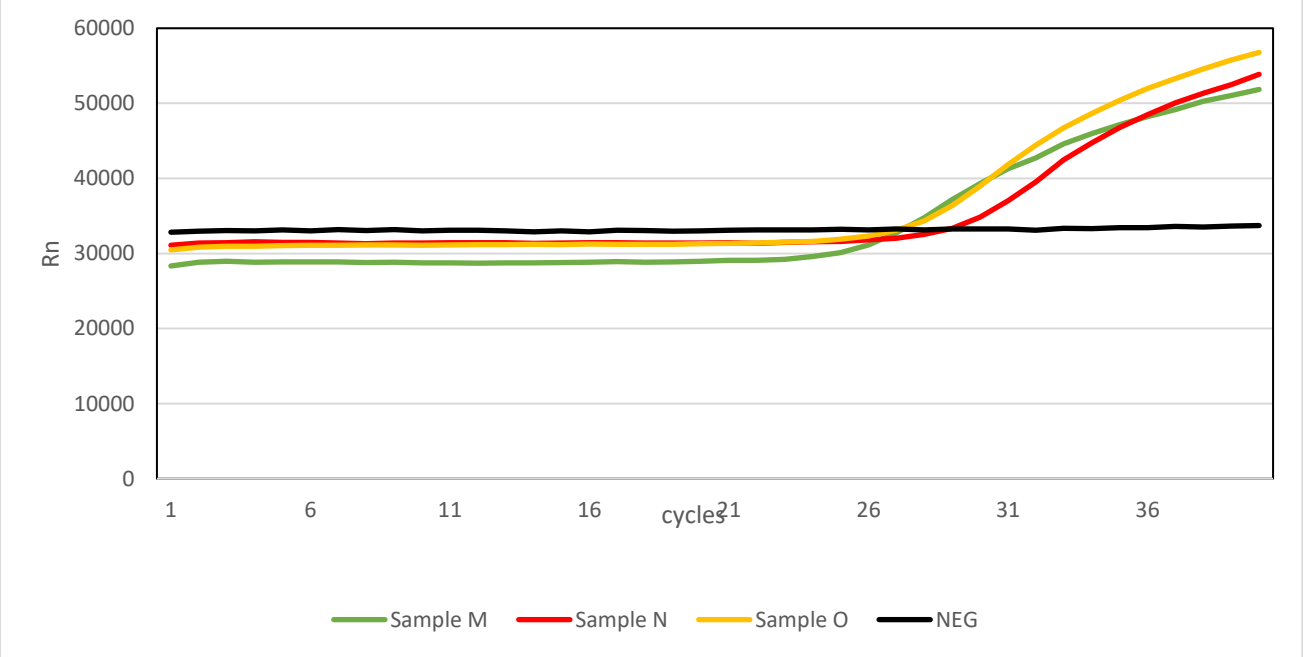

Figure19: Amplification plot of PCR by Histone(h2b) bone marrow

4.4.2 PCR Efficiency

PCR efficiency is a ratio calculated by taking the number of amplified target DNA molecules at the end of the PCR cycle divided by the number of DNA molecules present at the beginning of PCR. Serial dilution of the sample is used for calculating the Efficiency. In this dissertation Samples E is dilutions are made as 1:10,1:100,1:1000,1:10000,1:100000 with one replicate each. PCR efficiency is calculated using the formula,

$$Efficiency\ (\%) = (10^{-1/Slope} - 1)\ x\ 100$$

The figure 20 shows the amplification plot by PCR on Sample E and its dilutions by Mitochondria cytb primers and probes.

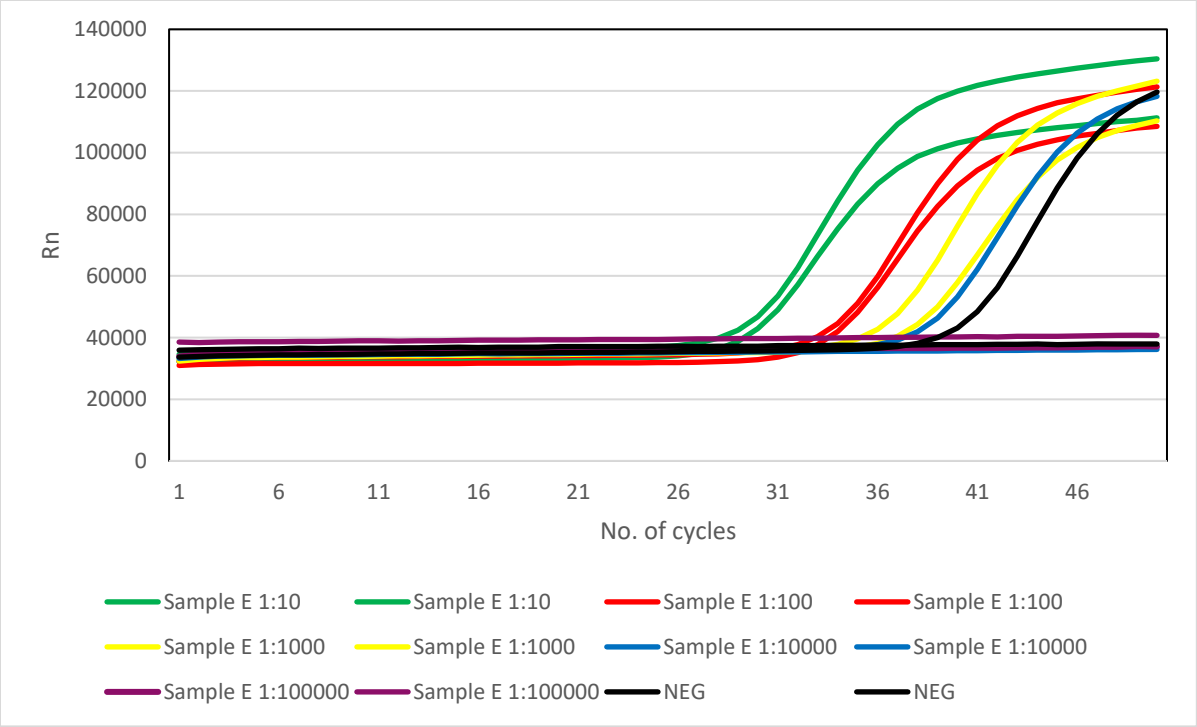

Figure 20: Dilution series By PCR on sample E on Mito cyt b primers.

The average Ct values (table 17) obtained from the amplification plot was used for calculating PCR efficiency.

Samples	Ct value (original)	Ct values (replicates)	Average Ct values
E 1:10	27.40838	27.59371	27.50105
E 1:100	31.36782	31.41323	31.39053
E 1:1000	35.6625	34.21812	34.94031
E 1:10000	35.4625	35.2351	35.354
E 1:100000	-	-	-

Table 17: Ct values of the dilutions

Dilution factor(table 18) is to be determined to find the slope of the curve. This is the chosen serial dilutions. In our case we used 1/10, 1/100, 1/1000, 1/10000. So, dilution factors were calculated as:

1/10 = 0.1

1/100 = 0.01

1/1000 = 0.001

1/10000 = 0.0001

Samples	Dilution factor	Log (10) dilution factor	Ct values average
E 1:10	0.1	-1	27.50105
E 1:100	0.01	-2	31.39053
E 1:1000	0.001	-3	34.94031
E 1:10000	0.0001	-4	35.354

Table18: Values for plotting standard curve

The above value in the table18 was used to make the standard curve. The average Ct value per sample and the log (10) dilution factor were used to calculate the slope and the R^2. An R^2 with a value close to one indicates the data follows a linear pattern.

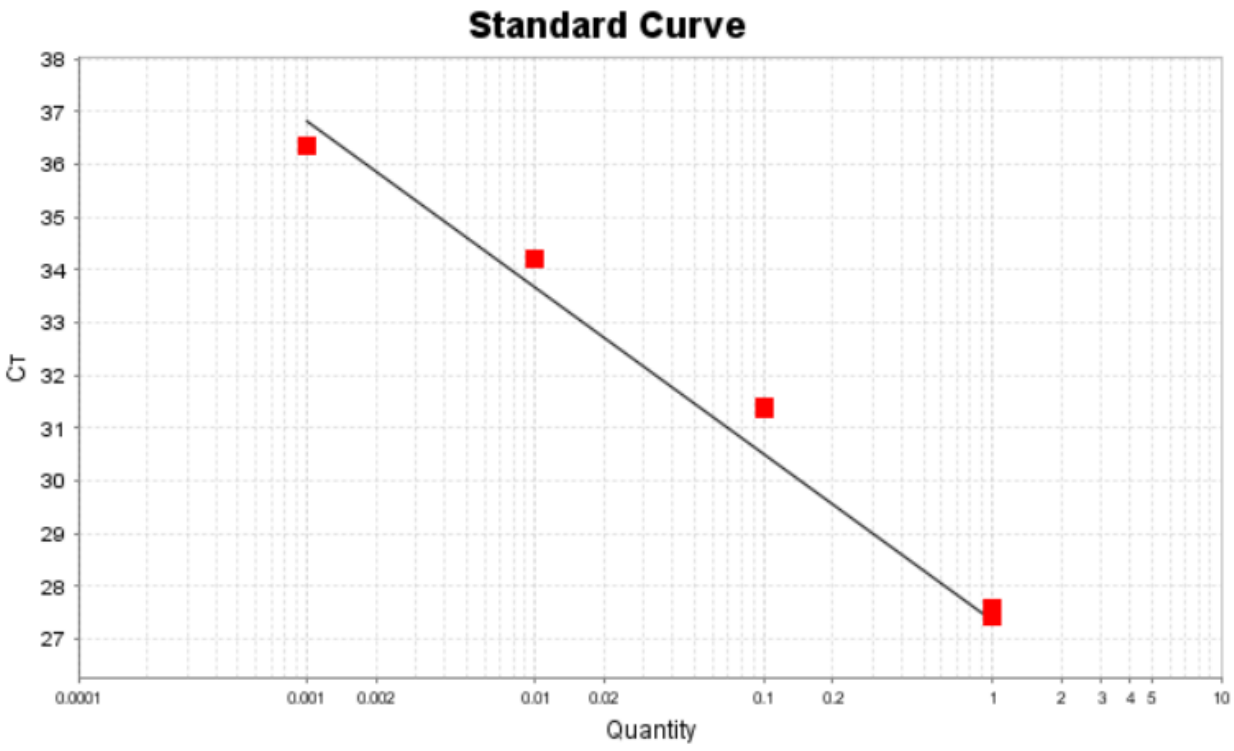

Figure 21: Standard curve obtained from the PCR reaction of serial dilutions on sample E.

From the figure 21, the slope = -3.156 and R^2 = 0.951. Slope value is used to calculate the PCR efficiency.

Efficiency (%) = $(10^{-1/-3.156}-1) \times 100 = 107.3995\%$.

The sensitivity was also focused on this study and accomplished by PCR method. Sensitivity is a strong approach for detecting the least concentration of copy number during multiplication. The sample E was serially diluted up to 10^5 and given in the figure 20. From the plot, it is understood that the least concentration of 10^4 could be amplified with the Cyt B primers. So, limit of detection in this experiment for PCR reaction was 10^4.

4.4.3 PCR specificity

PCR Specificity test was done by mitochondria cytb primers on two Pike samples and eight other fish samples with two negative controls amplification plots were shown in figure22. This test confirms that, the constructed Cyt B primers perfectly active towards the *Esox Lucius* species since only pike samples were true positive and the rest of the samples were true negative. Since, there is no false positives the specificity of the primers is 100%.

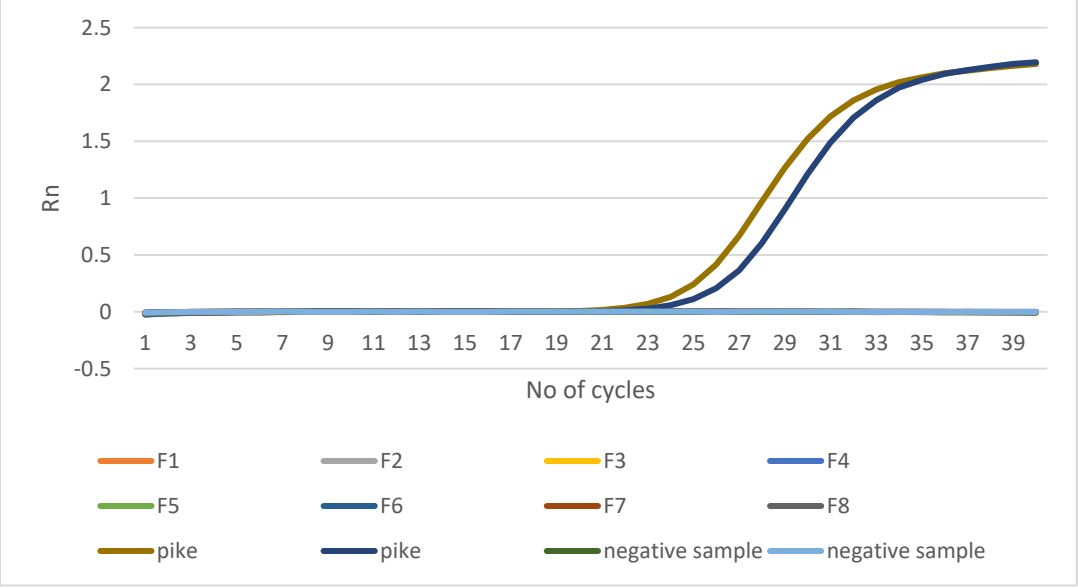

Figure 22: PCR Amplification plot of eight different species against mitochondria cyt b primers

The eight foreign samples along with Esox Lucius was tested and only the pike samples got positive amplification. Other samples were not amplified which proves the specificity of the biomarkers designed against the Esox Lucius histone genome which is shown in figure 23.

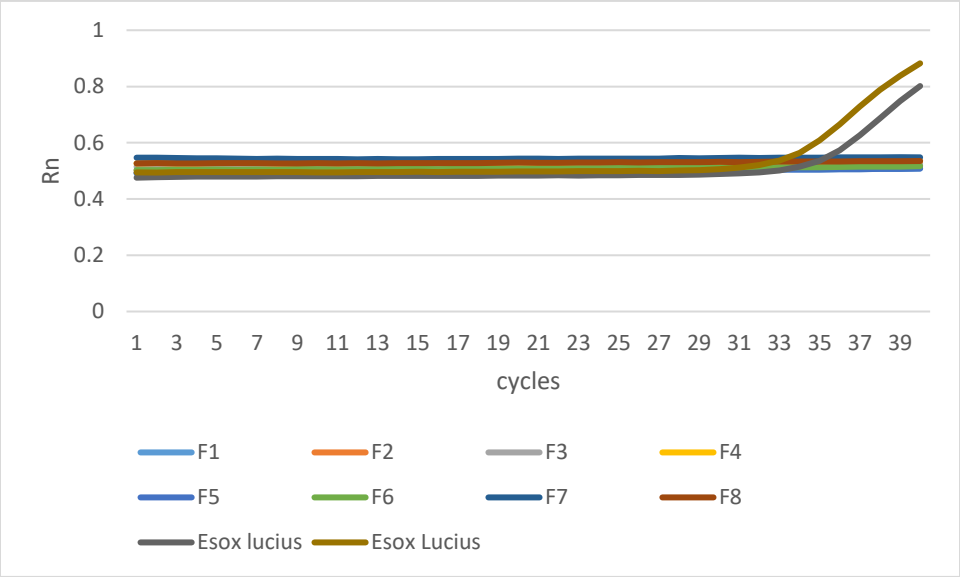

Figure 23: PCR amplification plot of eight different species against Histone(h2b) primers

Specificity test of GAPDH primers was done with five different kind of fishes.Among five fish species, only pike sample amplifies seen from figure 24 and reveals the specific gene of expression (GAPDH). Negative control also evidenced as staright line, which confirms the pike specificity.

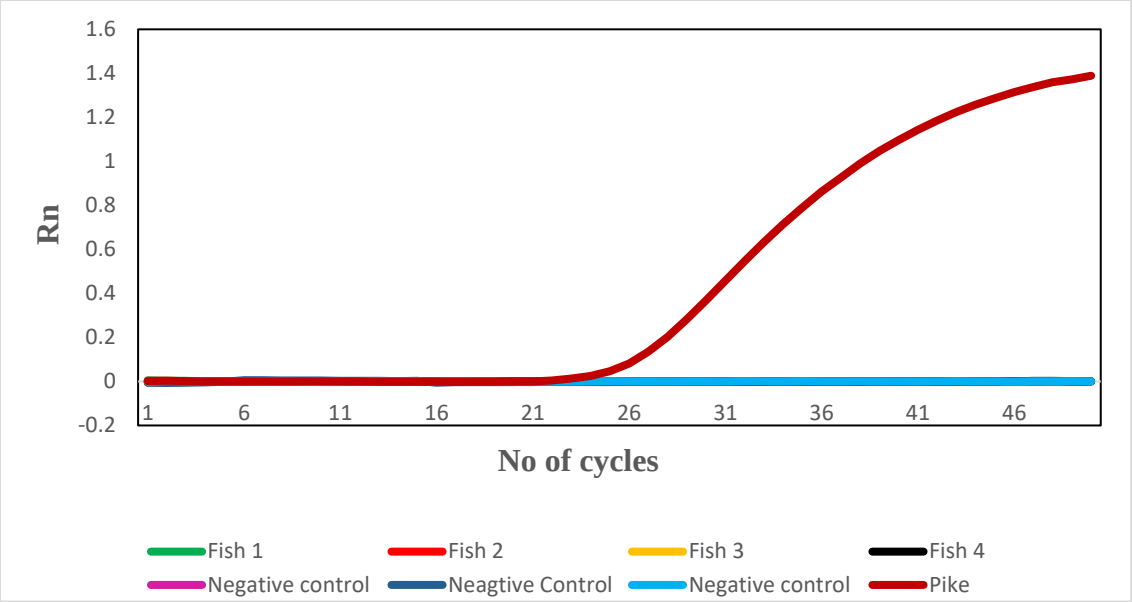

Figure 24: The specificity test performed by PCR with GAPDH primers.

The above results shows that all three gene of interest were 100% specific with the primers and probes designed.

4.4.4 Oligos testing for PCR

PCR was done for the positive oligos of Histone gene along with the developed primers set and shown in the figure 25. In addition, the dilution series of positive oligos from 10^1 to 10^6 were performed by PCR to analyze the least concertation of detection i.e., sensitivity. One replicate of each sample is added to each dilution.

The exponential phase in the graph follows the dilution pattern, which is a significant result. It shows that the PCR primers and probe designed for Histone was working well against the oligoes for Histone. The early Ct values shows the Oligoes were made perfectly to provide the optimum amplification results.

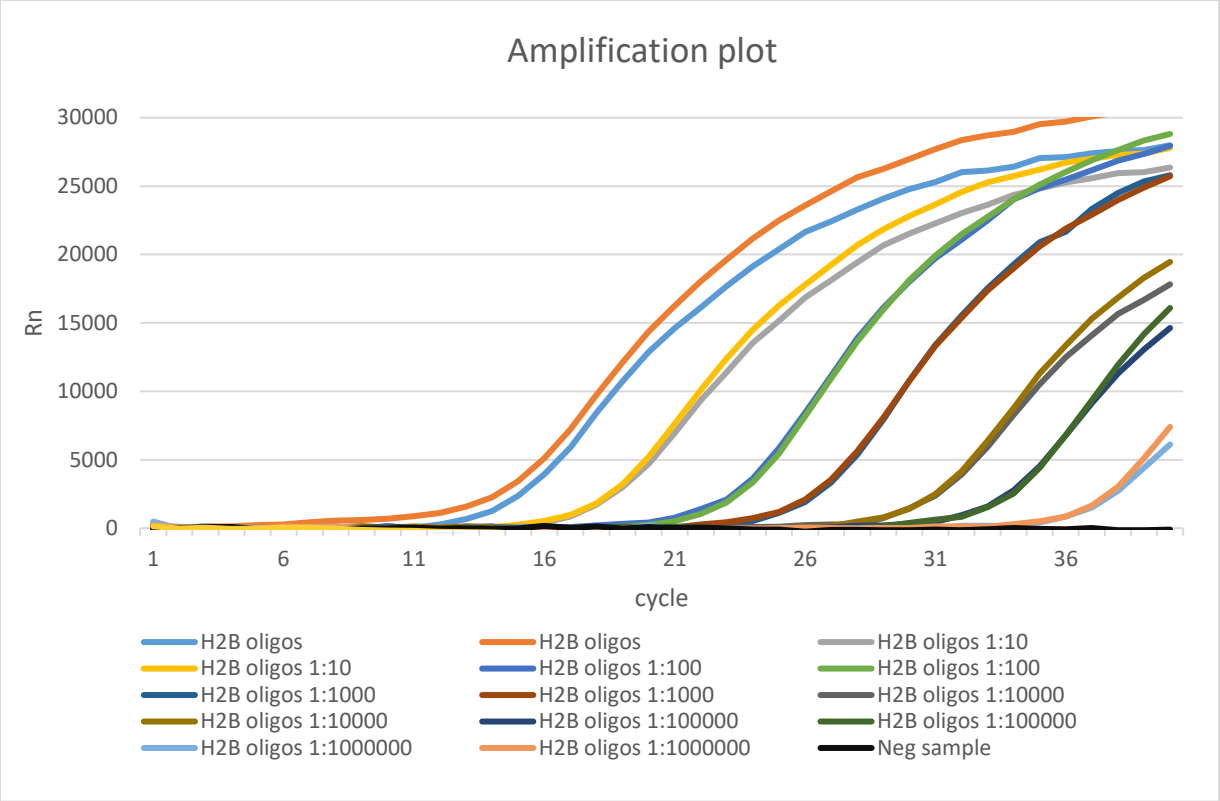

Figure 25: PCR on oligos with Histone(h2b) primers and probes

The figure 26 shows the amplification plot for oligoes done on esox lucius by GAPDH and it exhibits that, the amplification is possible until 1: 10^6 dilution. The maximum and minimum concentration starts the amplication cycle at 10 and 31, respectively. The initial concentration of GAPDH oligos is found to be 62300000 molecules/2.5µL and it is serially diluted upto 10^6, which consist of 62 molecules/2.5 µL. So, with least number of 62 molecules could be able to amplify with this appropriate reaction mix.

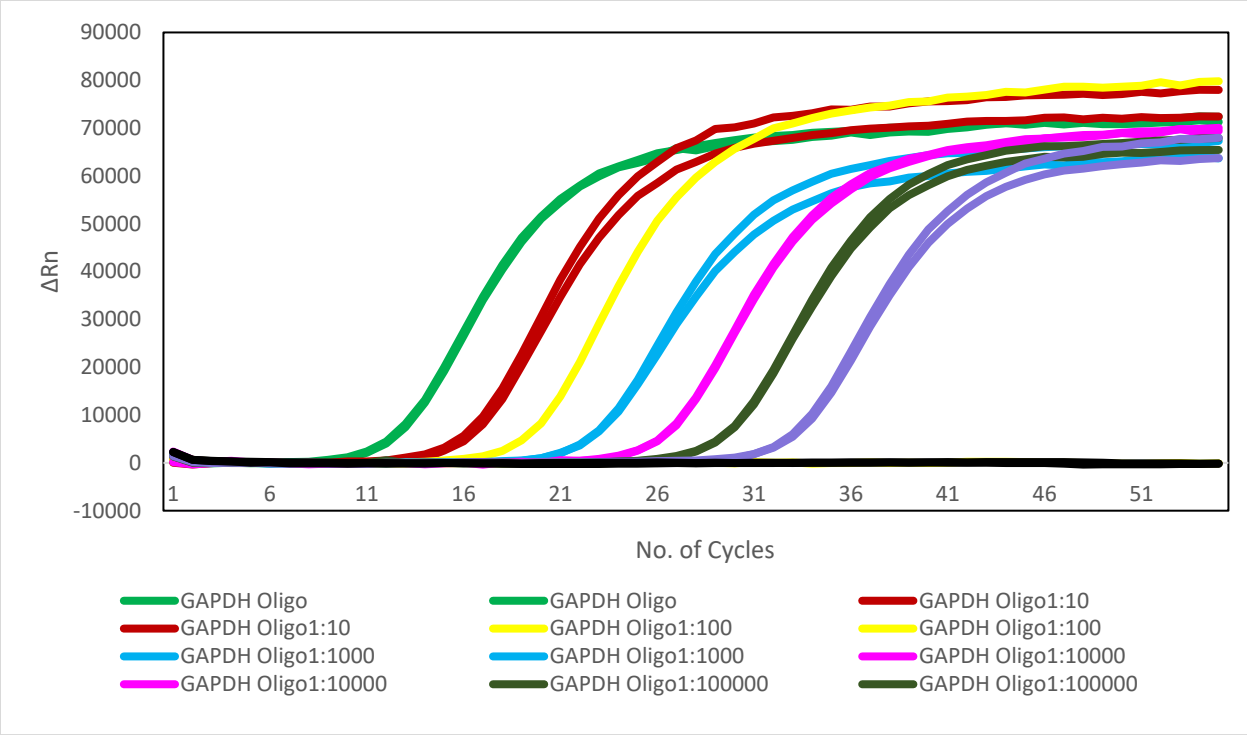

Figure26: PCR evaluation of serially diluted Oligos by GAPDH primers and probes

4.5 LAMP on Tissue

Four samples E, F, G and H from four different tissue regions of Esox Lucius observed to give amplified curve which proves the output to be positive. The test has been considered successful with the help of two negative controls which provide a flat graph or negative result which can been easily identified from the figure27. The method was optimized by real-time monitoring of the time and temperature of reaction. The optimal reaction temperature and time for the LAMP assay was proved to be 65 ◦C and 30 min, respectively. One cycle in the amplification plot represents 30 seconds which means the amplification starts between 36-46 cycles, that is within 18-23 minutes the LAMP reaction detects the targeted DNA.

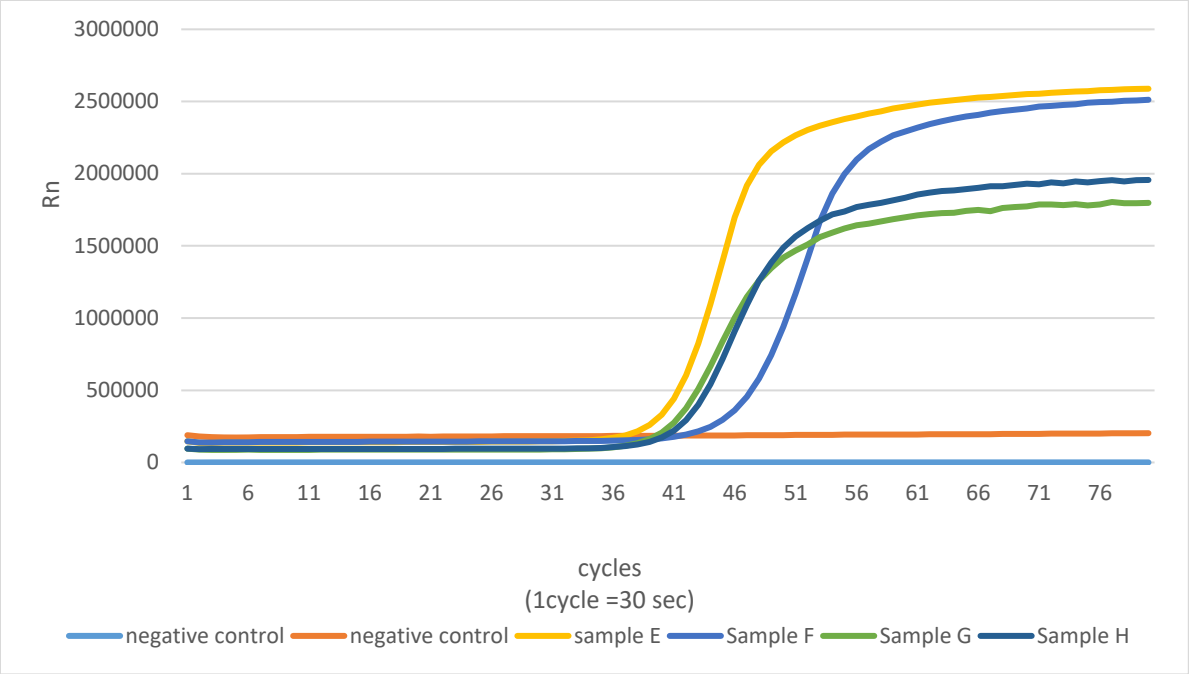

Figure 27: Amplification plot of LAMP reaction by mitochondria cytb on tissue samples

Amplification plot for LAMP Reaction by Histone primers shown in figure 28 where all the samples tested produced a negative result. The flat line tells us that there is no Amplification for the LAMP reaction on Histone gene. This means designed primers and probes failed to bind with the Targeted DNA template.

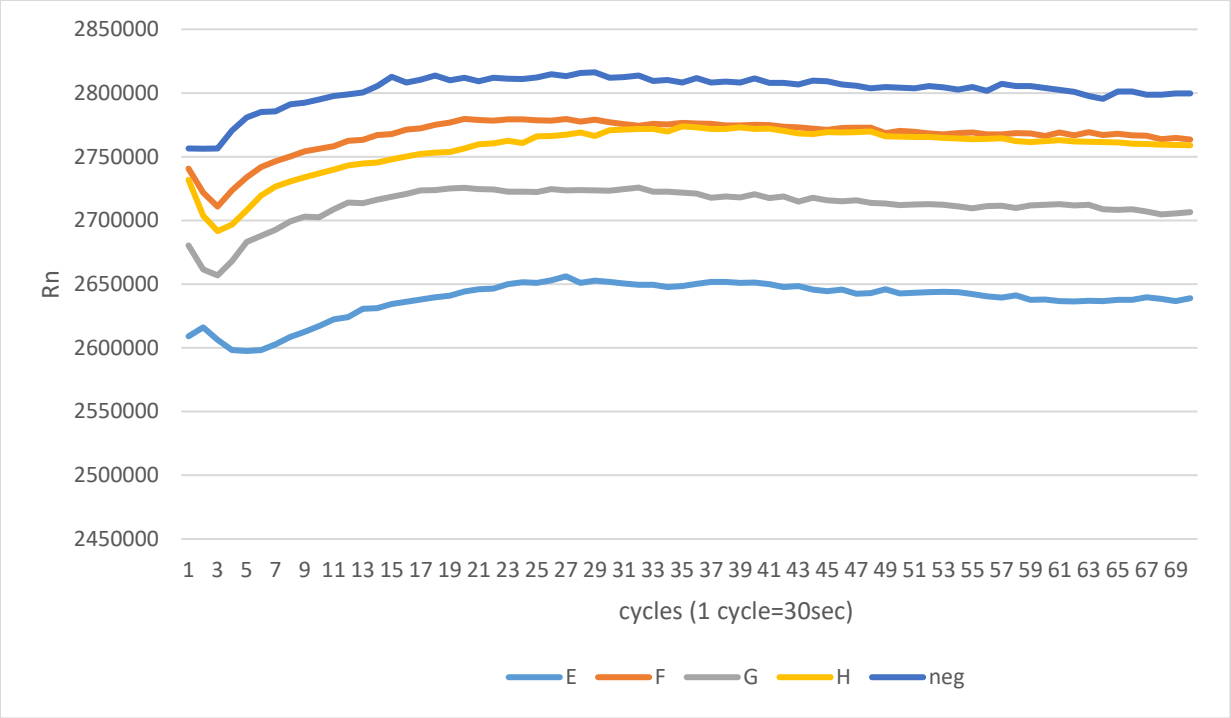

Figure 28: Amplification plot of LAMP reaction by H2B primers on tissue samples

The designed primers and probes for LAMP reaction on GAPDH was successful with the Tissue sample. Two samples E and F were tested against these primers and got an amplification within 23 minutes of the reaction process. A negative sample was also tested along with E and F to prove the integrity of the results showed on figure 29.

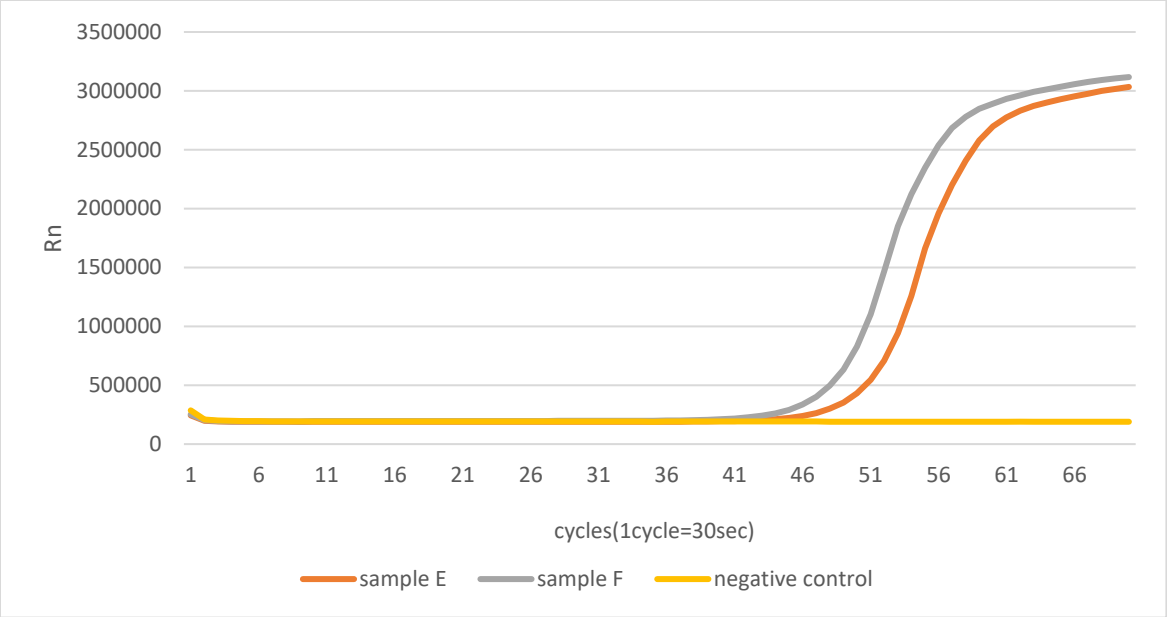

Figure 29: Amplification plot of LAMP reaction on GAPDH on tissue samples.

4.5.1 LAMP on Bone Marrow

Samples from bone marrow against mito cytb and GAPDH primers and probes tested positive which can be seen by the amplification of the curves from the figure 30 and 31 respectively. Mito cytb primers and probes bind the DNA much earlier than GAPDH. Amplification by mitochondria primers was within 14-16 minutes after the reaction started. While the GAPDH provided the positive result after 20 minutes. This result may be due to the less amount of DNA in the one of the samples.

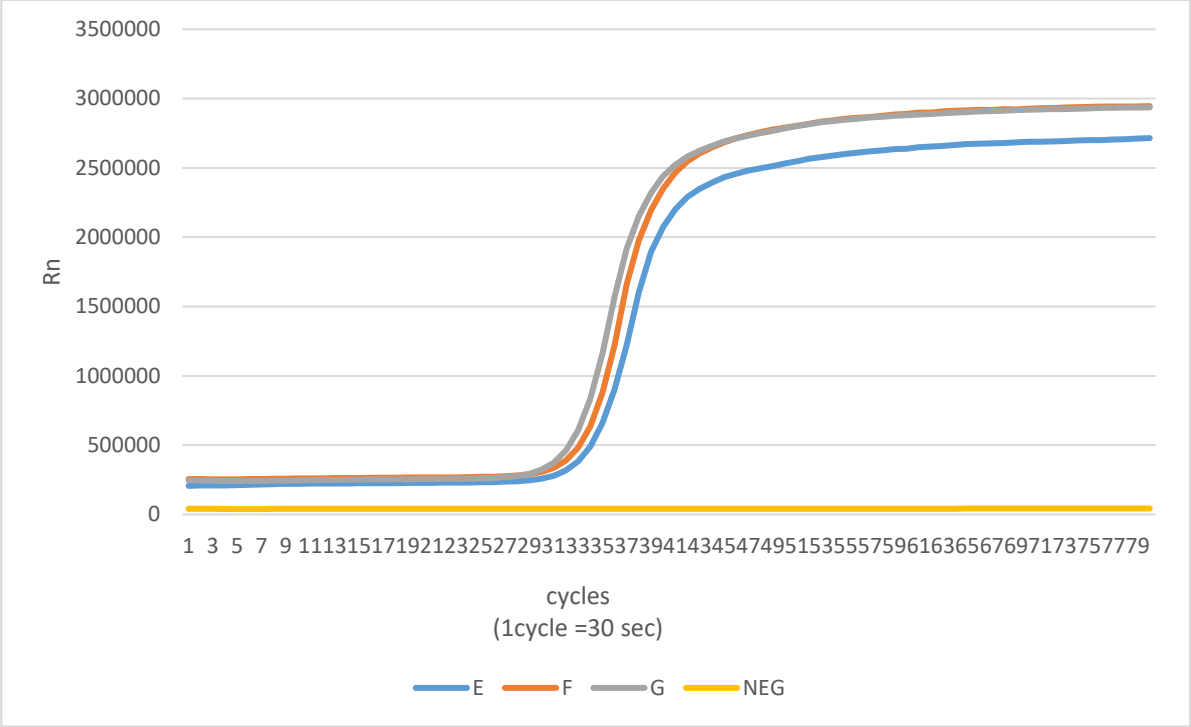

Figure30: Amplification plot for LAMP on mitochondria primers against bone marrow samples

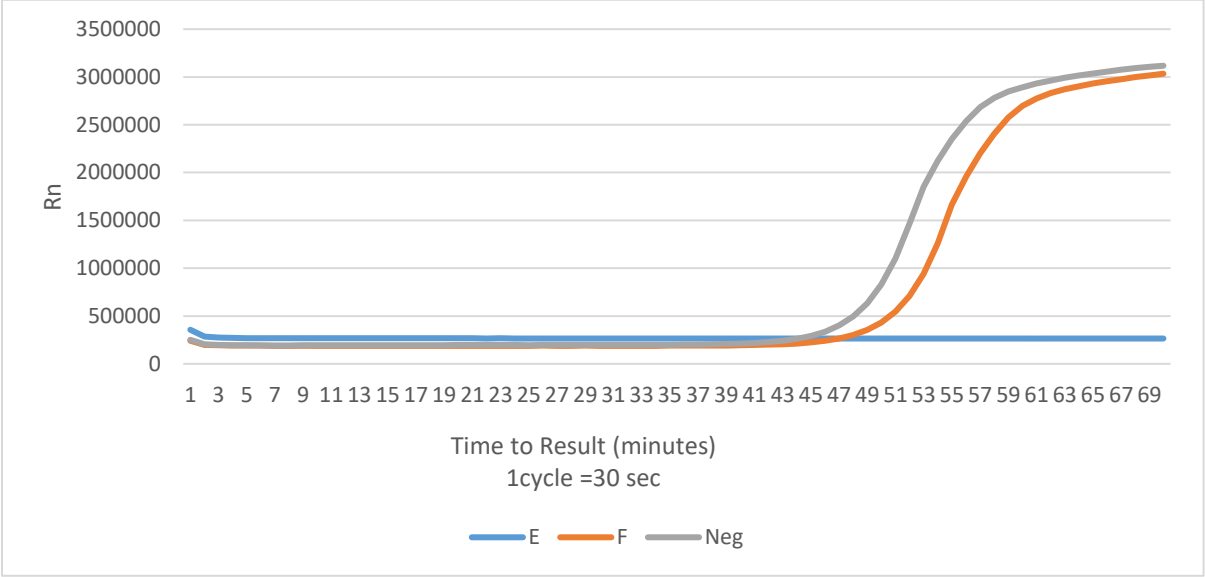

Figure31: Amplification plot for LAMP on GAPDH primers against bone marrow samples

4.5.2 LAMP sensitivity

Here we are analyzing the sensitivity by finding the Limit of Detection of the serial dilution made for LAMP reaction. The sample E was serially diluted up to 10^5 and given in the figure (32). From the plot, it is understood that the least concentration of 10^4 could be amplified with the Cyt B primers.10^4 is taken as least concentration since 10^5 were negative results. That is limit of detection of the LAMP reaction for the primers and probes created for cytb is 1:10^4.

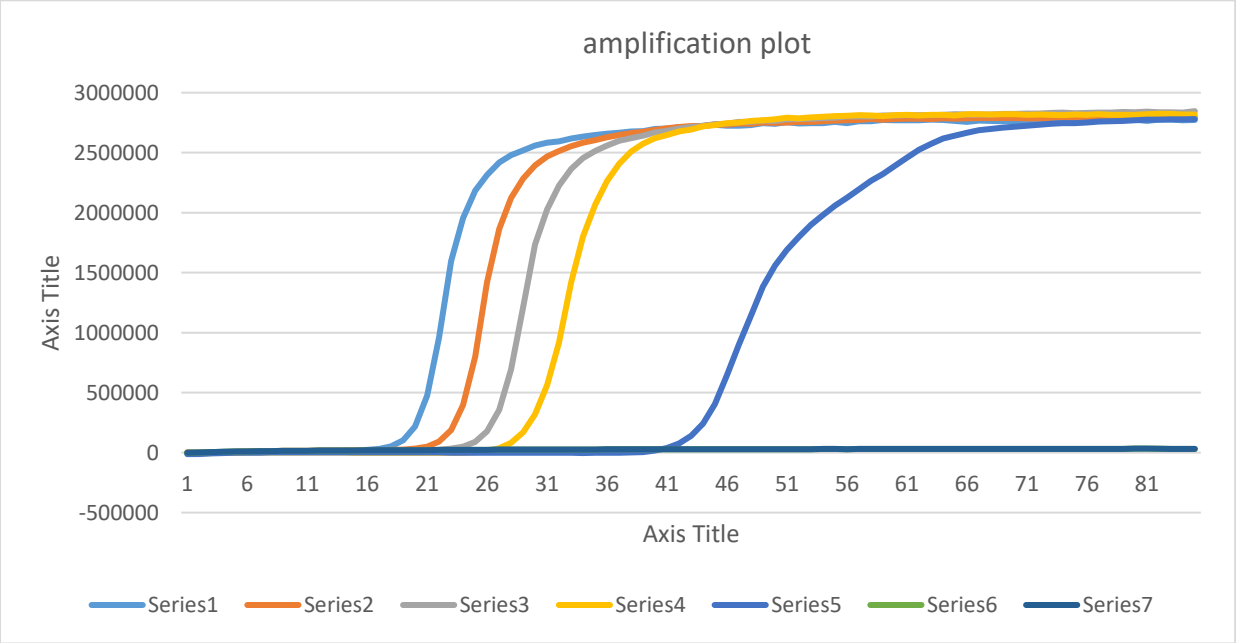

Figure 32: LAMP dilution series with sample E on Mito cyt b

4.5.3 LAMP Specificity

LAMP Specificity test was done for pike along with eight other fish species and two negative controls are shown in the figure (33). This test confirms that, the constructed Cyt B primers perfectly active towards the *Esox Lucius* species.

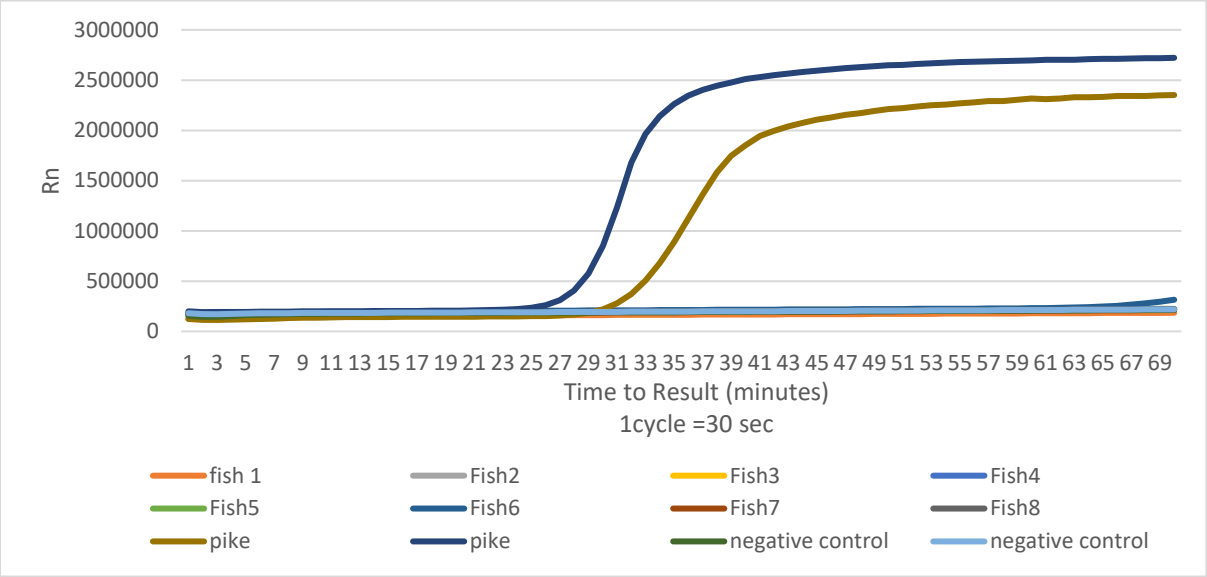

Figure 33: LAMP Amplification on different species by Mito cytb primers for testing specificity

The figure (34) shows the specificity test result for LAMP by GAPDH primers. Among five fish samples, only pike samples amplify and reveals the specific gene of expression (GAPDH). Negative controls also evidenced as straight line, which confirms the pike specificity by GAPDH primers and probes.

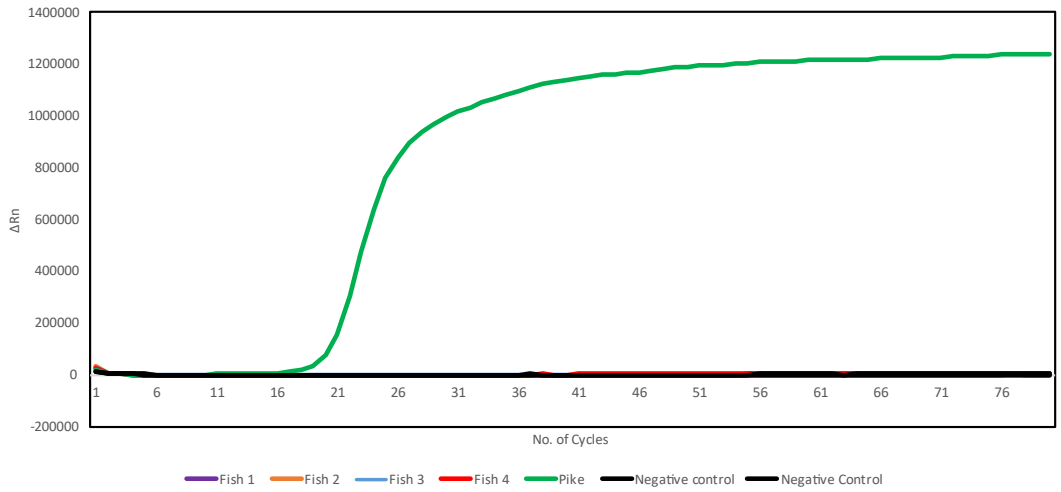

Figure 34: Specificity test amplification plot by LAMP reaction on different species by GAPDH primers

4.5.4 Oligos testing for LAMP

The Oligos testing against histone primers failed to get an amplification which is observed from the figure 35.

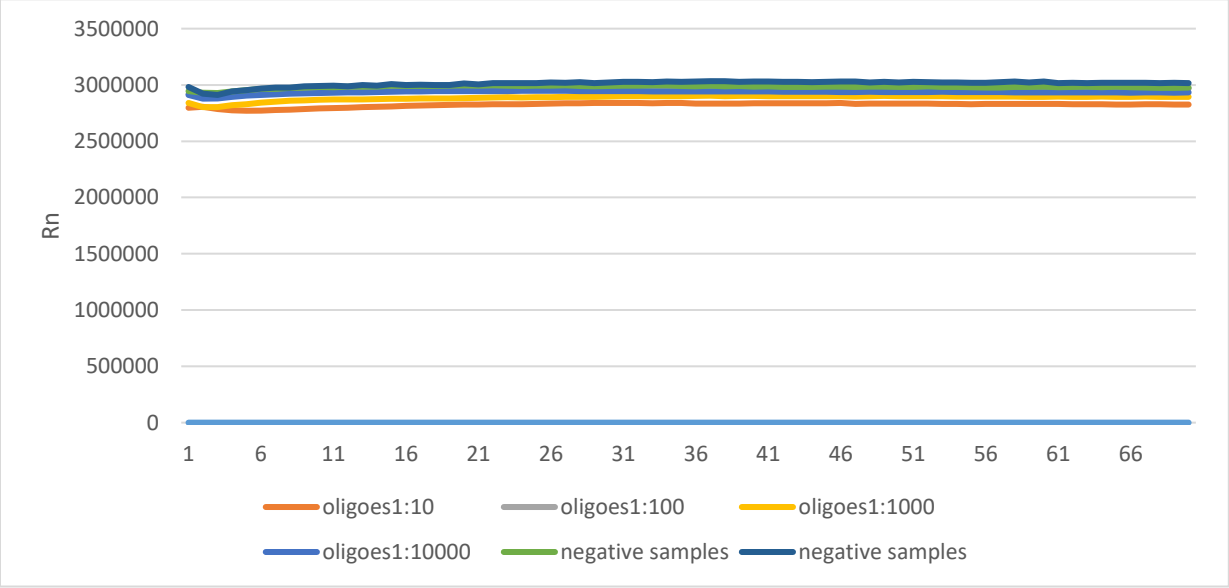

Figure 35: Amplification plot of Oligoes testing lamp Histone primers

Figure 36 shows the LAMP evaluation of serially diluted GAPDH positive oligoes, along with negative control. It exhibits that, the amplification is possible until 1:10^6 dilutions. The maximum and minimum concentration starts the amplification cycle at 21 and 56 respectively. The initial concentration of GAPDH Oligos is found to be 24900000molecules/ µL and it is serially diluted upto 10^6, which consist of 24 molecules/ µL. So with least number of 24 molecules culd be able to amplify with this appropriate reaction mix.

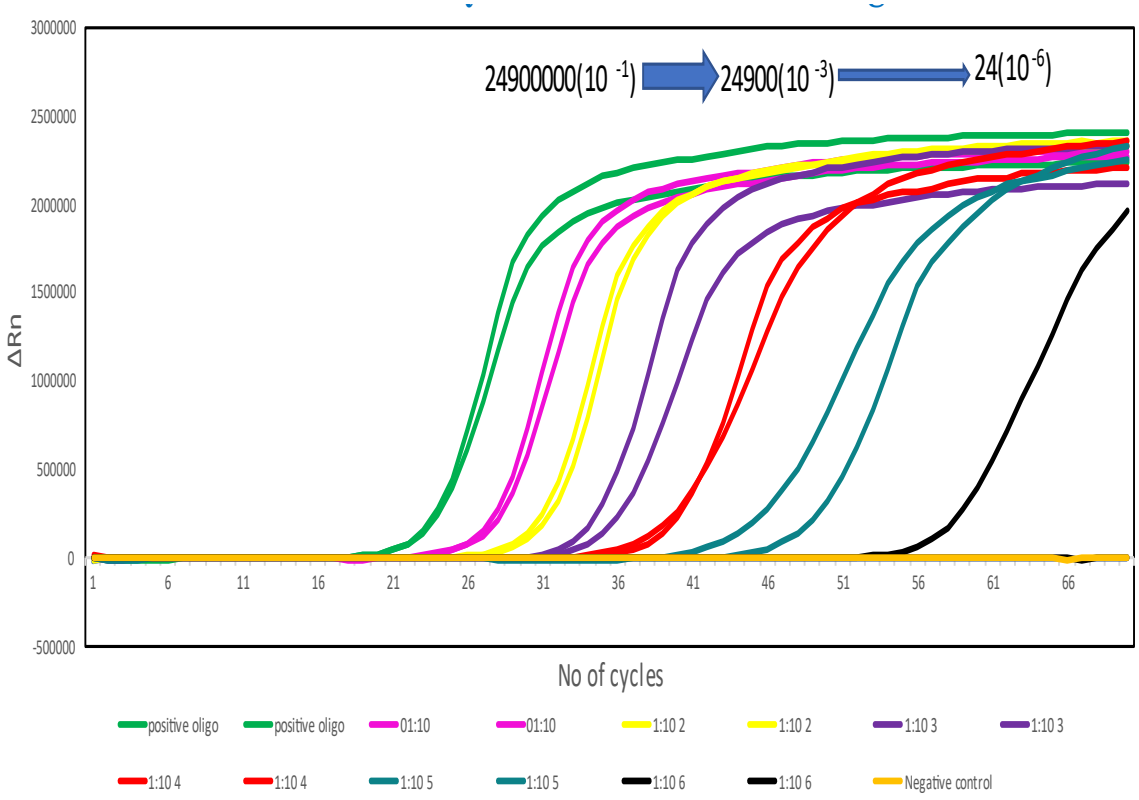

Figure 36: Amplification plot for Oligoes testing against GAPDH primers for LAMP Reaction.

4.6 NASBA on Tissue

Samples A1, A2, A3 and A4 tissue samples and negative samples A5and A6 that tested against Mitochondrial cytb primers were unsucessful. Amplification plot for NASBA tested along mitochondria cytb primers on tissue is shown in the figure 37.

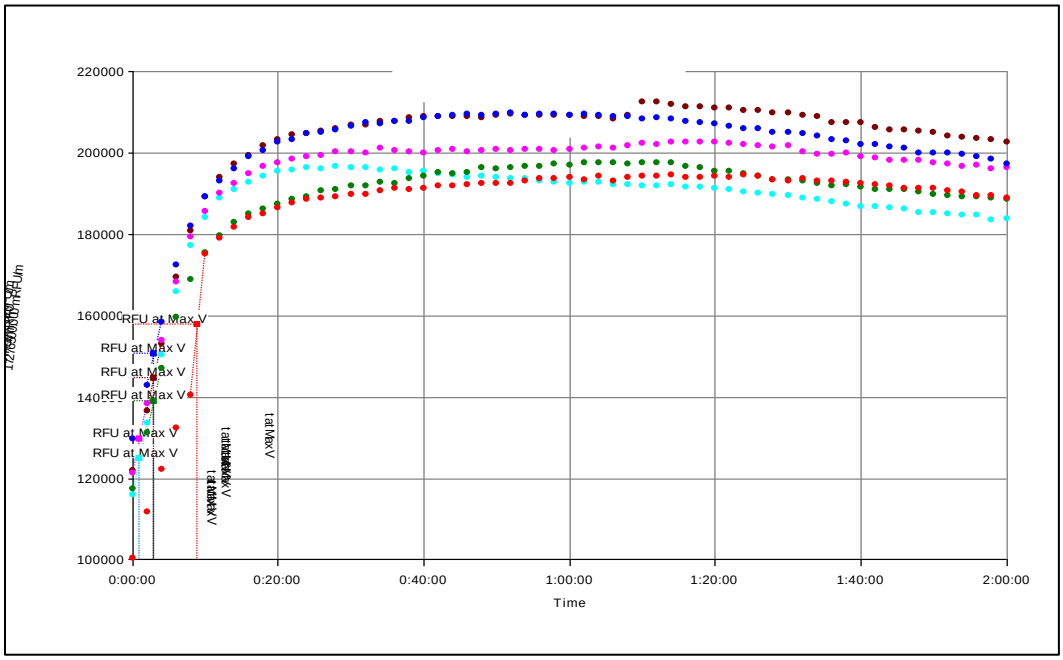

Figure 37: Amplification plot for NASBA with mitochondrial primers on tissue

Similarly eight samples A1,A2,A3 and A4 along with its 1:10 dilutions C1, C2, C3 and C4 respecctively tested against histone-h2b primers were failed to produce an amplification which was seen from figure 38.

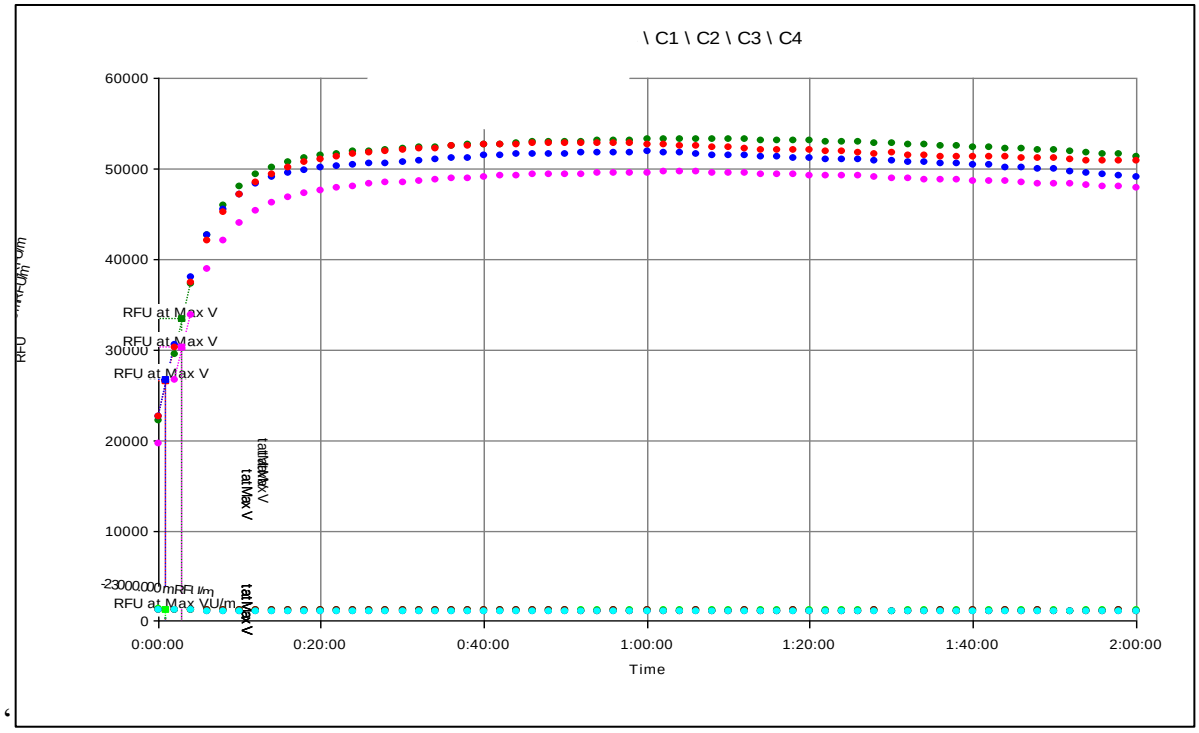

Figure 38: Amplification plot for NASBA with Histone primers on tissue

Four pure Tissue samples A1, A2, A3 and A4 that were tested against GAPDH were Positive and amplification plot is shown in figure 39. From the figure 39, it is clear that all the four pure samples give a sigmoidal shape which proves the positivity of the reaction. It gives a fluorescence value up to 110000 and amplification starts between 20-30 minutes.

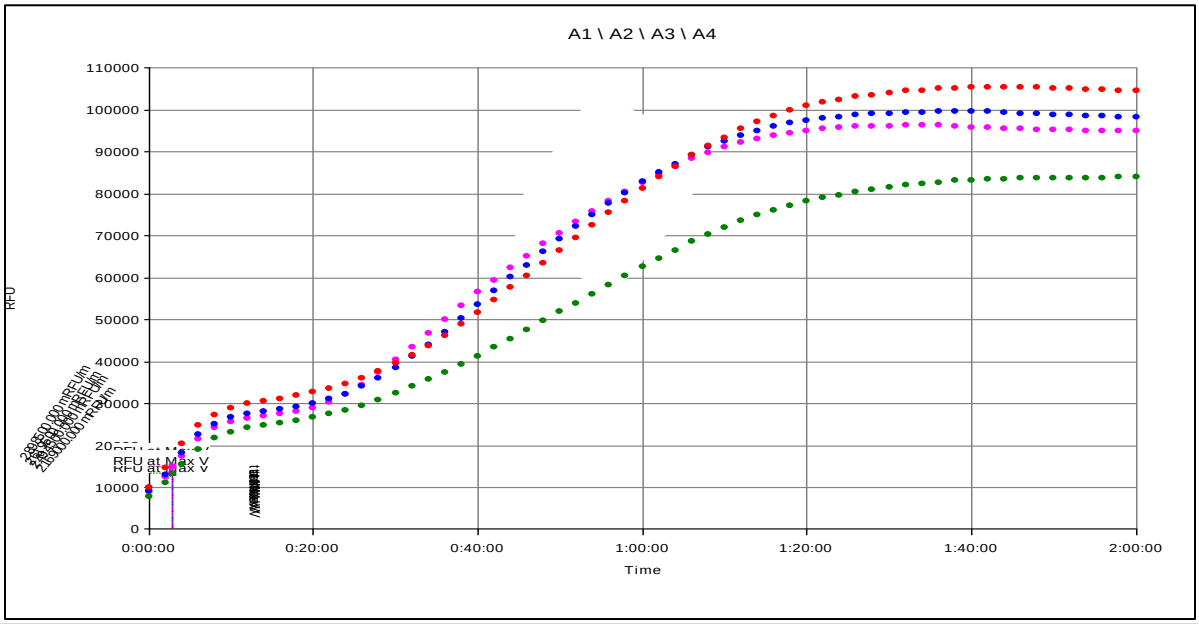

Figure 39: Amplification plot for NASBA with GAPDH primers on tissue

4.6.1 NASBA on Bone Marrow

Six bone marrow samples A1, A2, A3, A4, A5 and A6 from Esox Lucius were tested for mitochondria and Histone samples and the resultant amplification plot is shown in figure 40 and 41 respectively. A7 and A8 were negative samples tested along the samples. Bone marrow samples showed negative results against Mito cytb primers and probes. Out of six samples tested against histone primers and probes only two samples got positive.

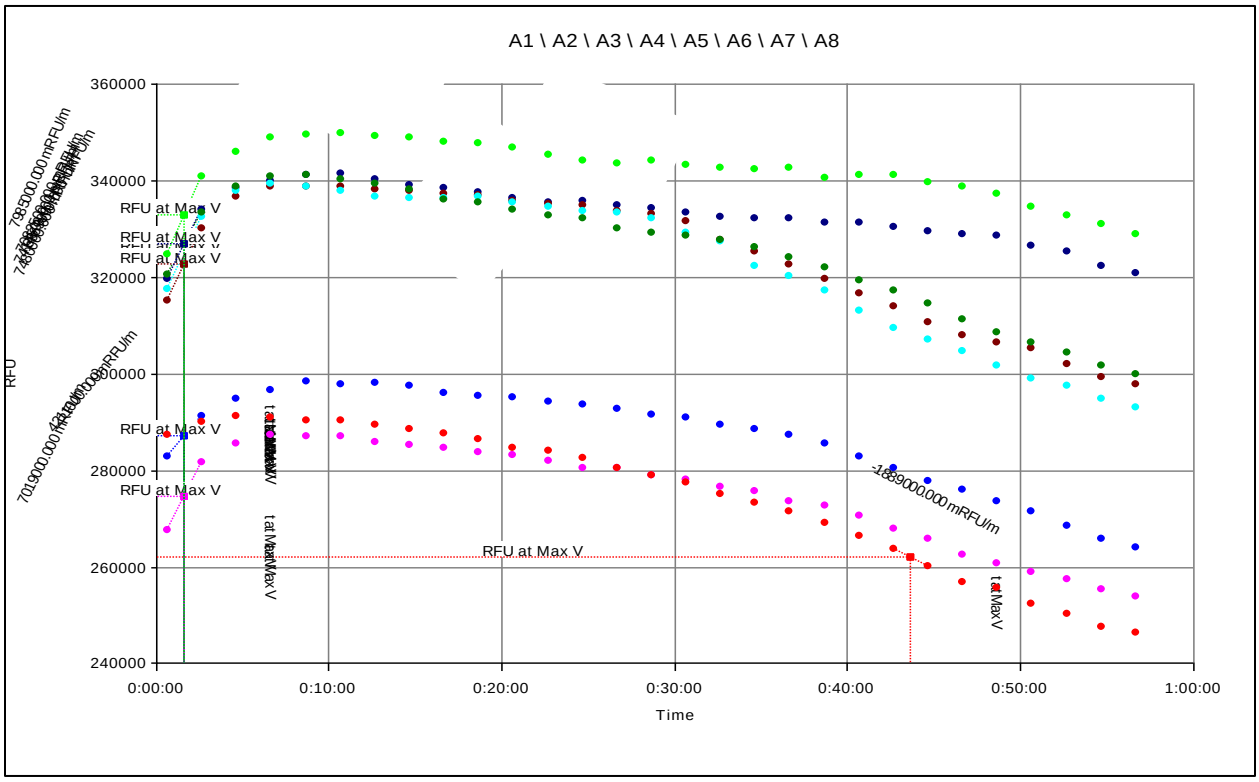

Figure 40: Amplification plot for NASBA with mitochondria primers on bone marrow

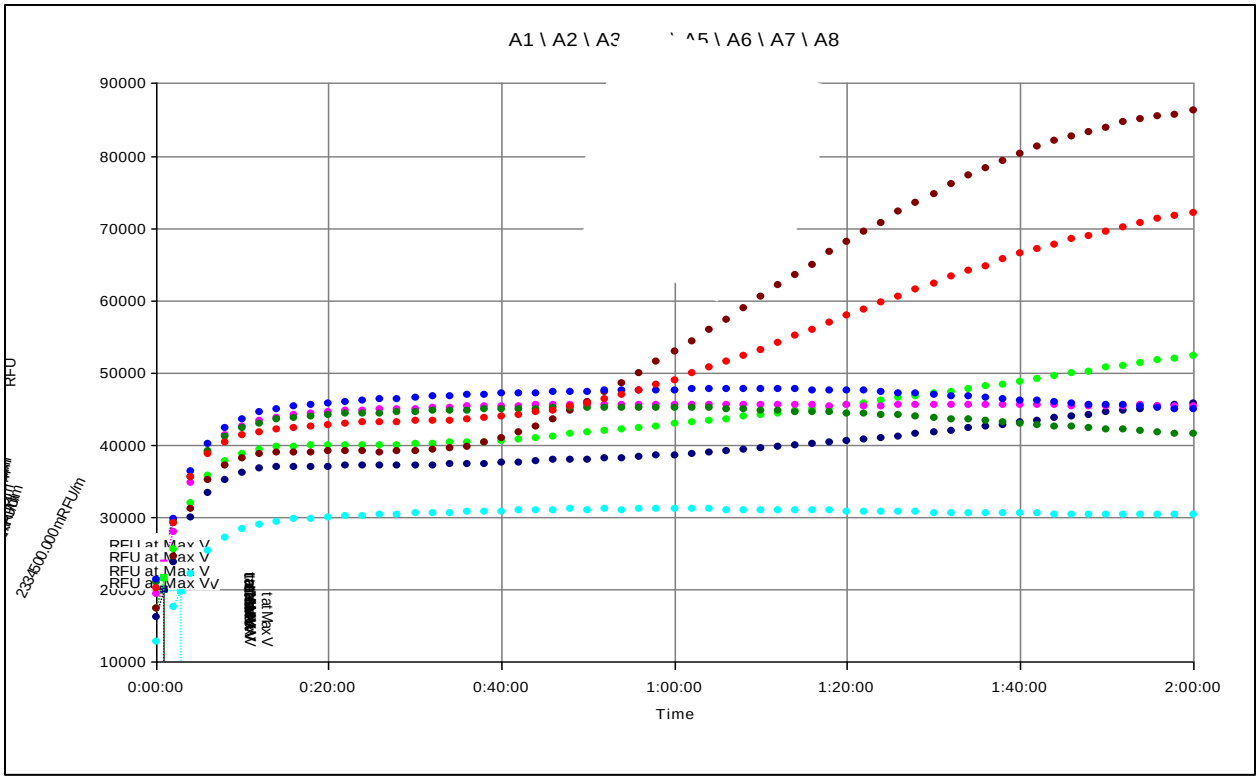

Figure 41: Amplification plot for NASBA with Histone primers on bone marrow

For bone marrow samples tested against GAPDH primers and probes provide with a positive result. Figure 42 shows the amplification plot of bone marrow sample named A1 against the GAPDH primers. The amplification starts at 20 minutes and ends at 1hour and provides a fluorescence value of 40000 to 110000. The ratio of the fluorescence value gives 2.75 which is greater than 1.7 shows the positivity of the reaction.

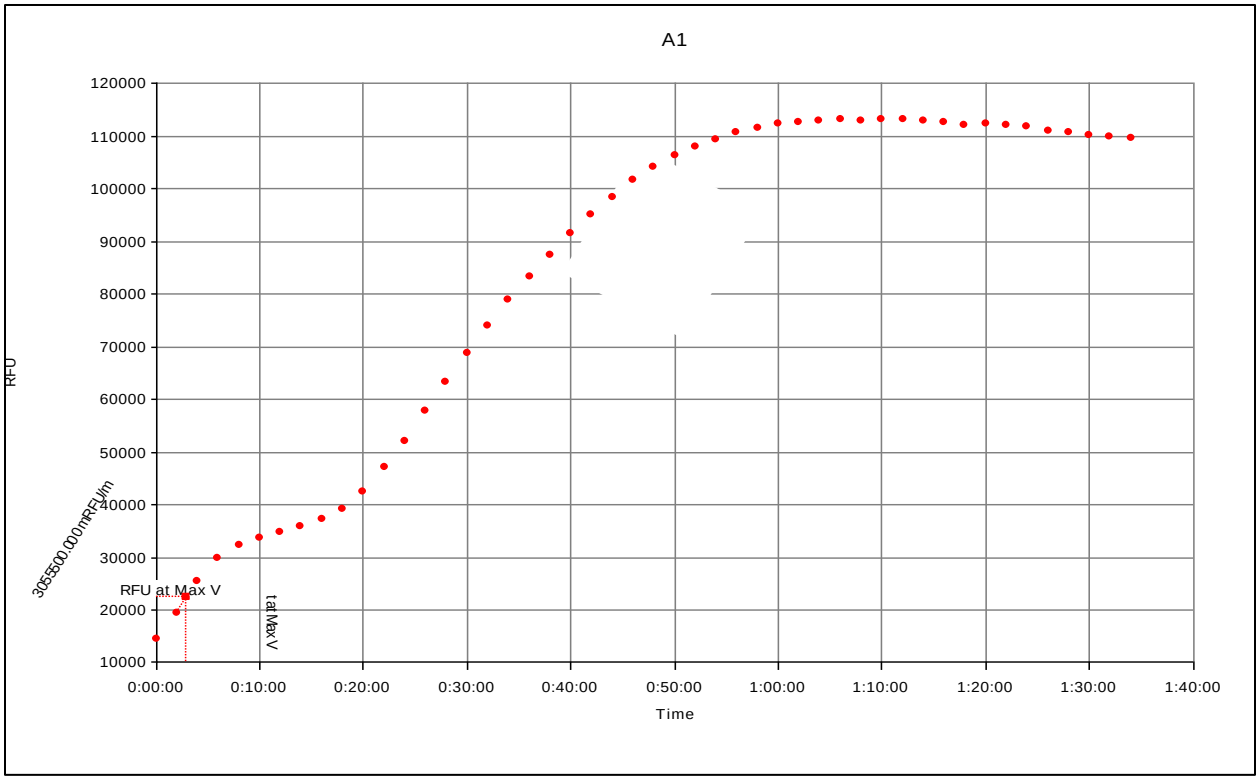

Figure 42: Amplification plot for NASBA with GAPDH on bone marrow

4.6.2 Oligoes testing for NASBA

NASBA was done for the positive oligos C1, C2, C3 and C4 against Histone primers set and amplification plot shown in the figure (43). Out of four samples only one sample showed positive result.

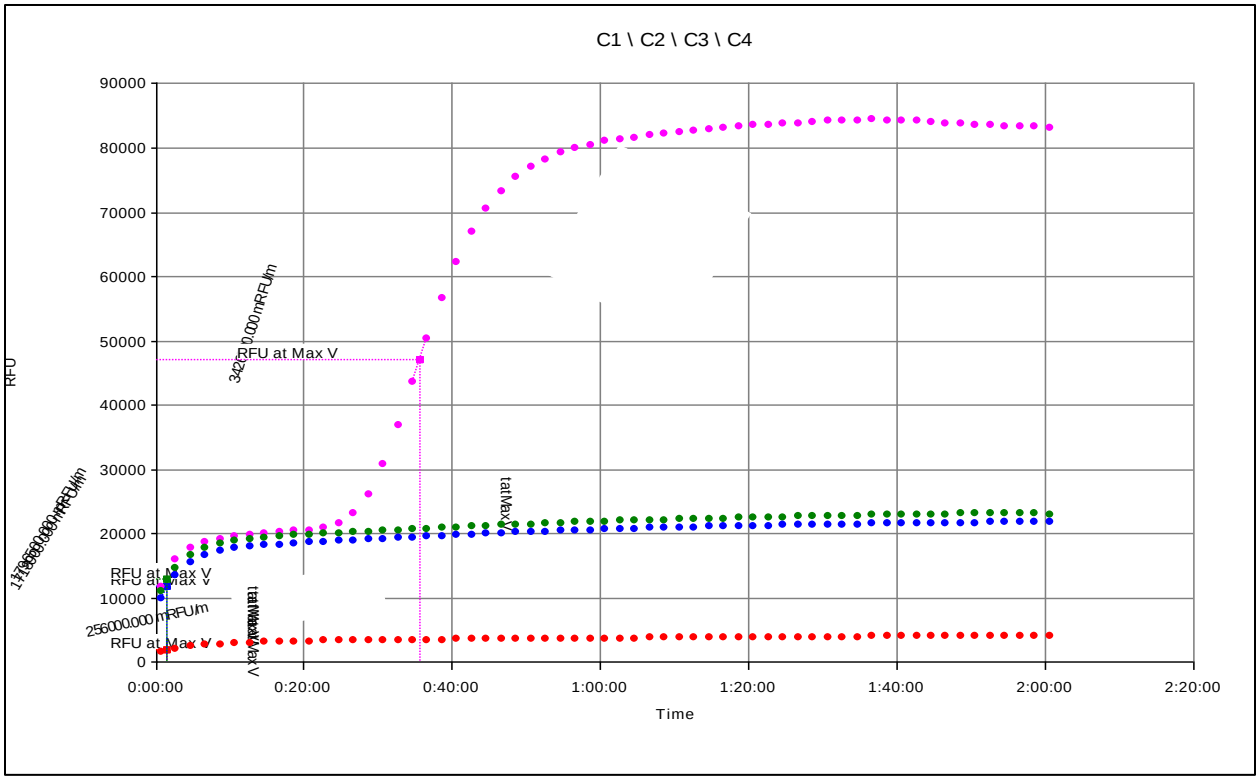

Figure 43: Amplification plot for NASBA with Histone primers on oligos

The figure 44 shows the amplification plot for oligoes done on esox lucius by GAPDH primers and probes and it exhibits that, the amplification is possible only for one sample.Other three samples tested along with it were negative.

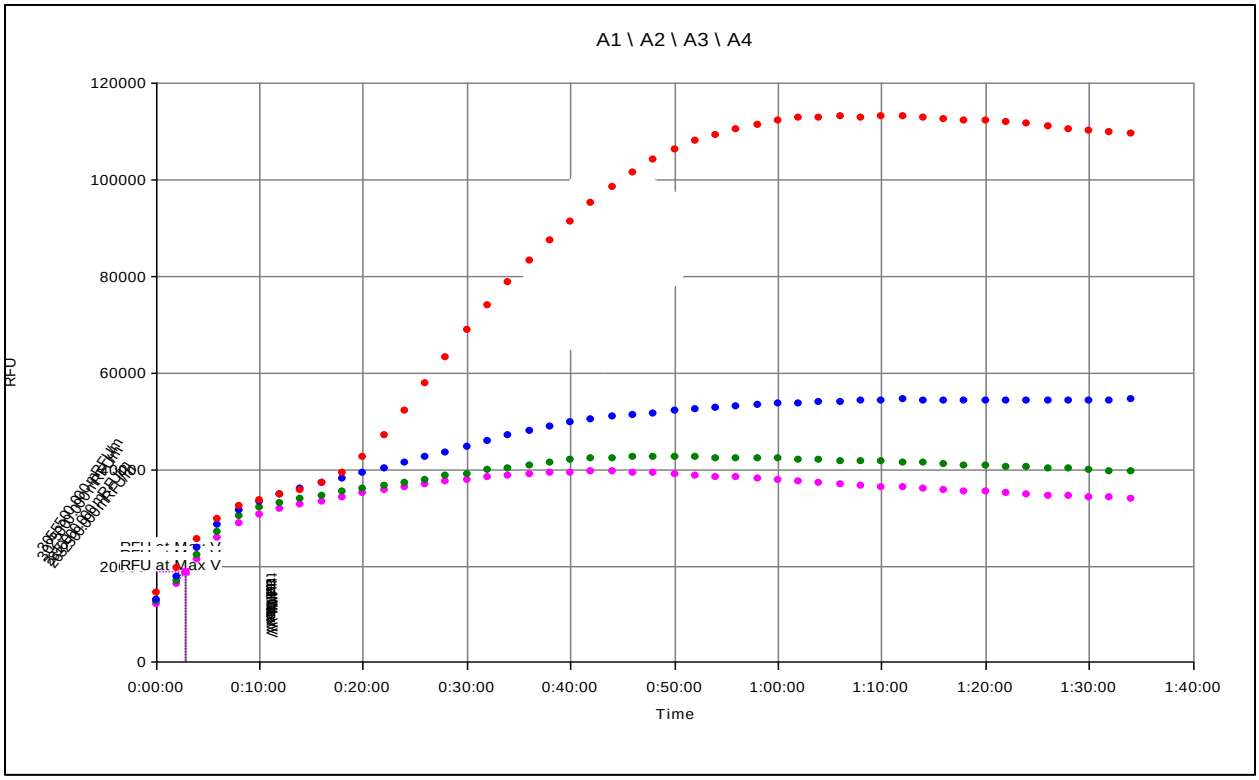

Figure 44: Amplification plot for NASBA with GAPDH primers on oligos

5.0 Discussion

5.1 Main Findings

This chapter is the discuss all my findings related to this thesis. The main task of this thesis was to detect eDNA from the fish name as PIKE in an environmental sample. The term environmental DNA (eDNA) refers to biological material or DNA that can be extracted from environmental samples. Analyzing DNA from water bodies is used for monitoring aquatic and semi-aquatic populations. The total eDNA present includes DNA that originates from different parts of animals and microorganisms occupying water bodies and similarly from animals that visit the environment, such as birds and mammals visiting the water body to drink [34]. In this project the experimental subject Esox Lucius was captured from two different Norwegian lakes. We collected the pike tissue, bone marrow, skin, and egg samples to prove the positivity of the test. However, the Tissue and Bone marrow samples amplification plot results are explained throughout the dissertation. Extraction of DNA was the first step of this thesis and the method used for the extraction of the DNA/RNA was BOOMS Extraction. This method was used for the extraction because it is very simple, rapid, reliable, and convenient for purification so many commercially available kits are used for this method [35. Throughout the extraction process a special precaution was needed in RNA free or a clean laboratory is needed for the extraction procedure.

The selection and design of Biomarkers are explained throughout the thesis. Various types of molecules, such as DNA (genes), proteins or hormones, can serve as biomarkers since they all indicate something about fish identity or their health. From the research I found that there are three important technical attributes: first, the biomarker must be present in peripheral body tissue and/or fluid (e.g., blood, urine, saliva, breath, or cerebrospinal fluid); second, it must be easy to detect or quantify in assays that are both affordable and robust; and third, it should be specific to reduce the false positive in the experiment.

The final part was focusing on the amplification and detection process of biomarkers with the three methods: PCR, LAMP and NASBA. Three different methods were compared to find the how they can detect e DNA, how different they detect different kinds of genes and how they can be correlated related to different amplification plots.

Three major part the results using PCR, LAMP and NASBA is done by correlation of different amplification curves. In general amplification plot tells the successful binding of the primers and probes and how the amplification process done and what kind of accuracy that had established us. to the targeted gene of interest.

The Table 14 and 15 shows an an overview of some of the results given by running PCR and LAMP respectively against 4 different PIKE tissue which is shown in Figure14 and 26. According to PCR protocol used in this experiment the reaction time for holding stage is 12minutes and each cycle completion needs 1.15 minutes. Now consider the number of cycles required for the amplification of each sample, for example 20 cycles = 12+20*1.15 min=35 minutes. It means the PCR requires 35 minutes for the amplification to start while LAMP reaction takes 19 minutes to start amplification. From these two Tables, it is observed that the PCR requires more than 2X time for getting results than a LAMP reaction.

species	Activity	Tissue sample	Dilutions	Cycles	Time for getting the result(minutes)
Mito cytb	PCR reaction	E	1:10	20	35
		F	1:10	25	40.75
		G	1:10	26	41.9
		H	1:10	27	43.05

Table 18: PCR amplification values from tissue sample

species	Activity	Tissue sample	Dilutions	Cycles	Time for getting result(minutes)
Mito cytb	LAMP reaction	E	1:10	38	19
		F	1:10	41	20.05
		G	1:10	39	19.5
		H	1:10	45	22.5

Table19: LAMP Amplification values from tissue samples.

Table 16 shows the overall result of each methods PCR, LAMP and NASBA. PCR and LAMP methods showed promising result during the whole project while NASBA showed only few positive results.

sample	Gene	PCR	LAMP	NASBA
Esox-Tissue	Mito	pos	pos	neg
	H2B	pos	neg	neg
	GAPDH	pos	pos	pos
Esox-Bone marrow	Mito	pos	pos	neg
	H2B	pos	neg	pos
	GAPDH	pos	pos	pos

Table 20: Comparison of output for PCR, LAMP and NASBA method.

Comparing the amplification plots of Tissue sample in figure 15,16 and 17 with figure 18 and 19 by bone marrow samples, it is clearly visible from the graph that the early Ct values are observed from the bone marrow samples amplification plot. From these figures it is evident that the DNA template in bone marrow is higher than that of the Tissue part. For a PCR reaction the bone marrow samples gave better result than the Tissue samples. Out of the three genes selected Histone (h2b) produced a late amplification. One of the reasons for this could be the low amount of DNA present in the extracted samples. Another reason could be the poor primer selection where the primers were not optimal for the binding of the sequence. Refer the figure 22, 23, 24, 33 and 34 the specificity of the primers was excellent as they didn't detect any false positives. PCR and LAMP test showed that the designed primers and probes of Histone and GAPDH were 100% specific to the experimental subject.

Oligos were tested to find out whether the designed primers are working or not. It also helps to find out the amount DNA template found in the extracted sample. For example, comparing figure 17 and 26 both use similar designed primer. The amount of DNA concentration in the sample is unknow on the figure17 and in figure 26 the amount of oligos concentration is known. Figure 17 produce an amplification on 26^{th} cycle. By analyzing figure 26, the amplification starting from 26^{th} cycle consists of 6230 molecules/2.5 µL which is equal to the figure 17. This is a way to find the DNA template concentration in any of the samples from Tissue and bonemarrow.Similarly ,in LAMP reaction by using oligos the concentration of DNA can be founded out.

NASBA experiment was more complex procedure out of three methods and the results were not good.

Mitochondria cytb designed primers and probes were not working with NASBA while GAPDH gave a sucessful amplification curve. HISTONE primers were active on bone marrow but not on tissue samples .Out of four oligos tested GAPDH and HISTONE primers only one sample in each reaction got postive.This may be due to lack of RNA templates in the sample or the poor designing of the primer. Most of the negative results shows that the need for improvement in the design of primers and probes to an optimum specifications. It also shows the failure of the denaturation of the DNA strand while the reaction process.

6.0 Conclusion and upcoming works

The main task of this thesis was to detect eDNA from the fish name as PIKE in an environmental sample was successful with the two methods PCR and LAMP while NASBA gave satisfactory result. This project has given a brief overview of the extraction of DNA, designing of the primers and the detection of DNA using three amplification methods PCR, LAMP and NASBA.

From this research, it is identified that both LAMP and PCR shows promising results during the experiment and NASBA showed satisfactory results. When compared to the amplification LAMP gave early results than PCR. It is difficult to establish good primers and probes for NASBA. The results show that it is easier to establish good LAMP and PCR primers and probes for different Pike biomarkers. Our primers and probes show that LAMP and PCR are two equally good sensitive and specific methods for being able to detect eDNA from Pike. We report here good primers and probes for the detection of Pike eDNA. Our selected NASBA primers and probes worked less satisfactorily and must be redesigned and further developed for them to be used for detection of Pike eDNA.

The thesis gives researchers a good possibility for future tech nano devices for finding the information from environmental species around the world.

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